



Differential Innate Immune Stimulation Elicited by Adenovirus and Poxvirus Vaccine Vectors

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Differential Innate Immune Stimulation Elicited by Adenovirus and Poxvirus Vaccine Vectors

A dissertation presented

by

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To

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Differential Innate Immune Stimulation Elicited by Adenoviral and Poxviral Vaccine Vectors**Abstract**

Vaccines are one of the most effective advances in medical science and continue to be developed for applications against infectious diseases, cancers, and autoimmunity. A common strategy for vaccine construction is the use of viral vectors derived from various virus families, with Adenoviruses (Ad) and Poxviruses (Pox) being extensively used. Studies utilizing viral vectors have shown a broad variety of vaccine-elicited immune response phenotypes. However, innate immune stimulation elicited by viral vectors and its possible role in shaping these vaccine-elicited adaptive immune responses remains unclear. Here we show that Ad and Pox vectors display profound intra- and inter-group differences in innate immune cytokine and chemokine elicitation. The CD46-utilizing vectors Ad35, Ad26, and Ad48 induced greater anti-viral and proinflammatory cytokines and chemokines relative to Ad5 in vaccinated rhesus monkeys and stimulated human PBMC. Ad fiber protein, as well as other capsid components, influenced resultant Ad vector innate stimulatory phenotypes. Analysis of human sera from Ad26-vaccinated volunteers showed similar anti-viral and proinflammatory cytokine and chemokine elicitation. Mechanistic analysis of Ad innate immune stimulation showed greater amounts Ad35 and Ad26, and small amounts of Ad5, traffic to the late endosome following infection. Innate immune stimulation by all three was reduced by inhibition of endosomal acidification, Cathepsin B, and Caspase-1, suggesting a common set of innate immune sensors triggered by Ads between 0-6 hours post-infection, in agreement with trafficking data showing Ad vector colocalization in the late endosome at similar time points. These data suggest a model mechanism explaining differences in observed Ad vector innate immune stimulation phenotypes. Similar to results obtained with Ad vectors, analysis of

innate cytokine and chemokine responses elicited by Pox vectors ALVAC, MVA, and NYVAC showed that all three were distinct, with the canarypox-based vector ALVAC eliciting a unique potent proinflammatory response. Together these results reveal surprising and pronounced differences in innate immune stimulatory properties of viral vectors. Furthermore, these results could lead to possible strategies for targeted construction of vaccines for desired innate immune phenotypes, and have profound implications on vaccine design against infectious diseases and cancers, as well as gene therapy.

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Dedication: I would like to dedicate this thesis to countless friends and dear family who have supported me over the years. I owe a tremendous amount of gratitude to each and every one of you. I would like in particular to dedicate this thesis to my parents, Fredrick and Marie Teigler. To my father, for teaching me from an early age to see the forest for the trees, and to my mother, for showing me what true perseverance and dedication is.

Chapter 1: Introduction

The scale and scope of HIV's medical and social impact has motivated the search for several approaches to treat or prevent HIV infection. Strategies to prevent HIV infection have included a broad variety of approaches such as antiretroviral drugs for pre-exposure prophylaxis (1), antibody expression via vectored immunoprophylaxis (2), penile circumcision (3–5), intravaginal microbicides (6), or anti-HIV vaccination (7, 8). Of these preventative approaches, a protective global vaccine against HIV holds the greatest promise of curtailing the pandemic due to low relative cost of and simplified public health implementation relative to other intervention strategies. Unfortunately, early attempts at vaccine design focusing on classical vaccine-production techniques such as surface protein immunization (9, 10) and viral attenuation (11–13) were found to be insufficiently immunogenic or unacceptably dangerous due to the possibility of viral reversion or neonatal pathogenicity. Additionally, viral inactivation of HIV virions for vaccine derivation is widely accepted to carry unacceptable risk due to the possibility of incomplete inactivation. Furthermore, HIV vaccine design has been frustrated by biological properties of HIV which make it recalcitrant to classical vaccine-elicited immune responses such as extensive viral diversity, the early establishment of a persistent cellular reservoir, and resistance to antibody-mediated neutralization. While the scope and timescale of difficulties in making an HIV vaccine is disconcerting, the unique challenge posed by making a protective vaccine against HIV has ushered in a new age of vaccine design with researchers searching for new ways to elicit stronger or unconventional immune responses against HIV. Despite a large number of pre-clinical and clinical studies having conducted for HIV vaccine design however, no correlates of immune protection against HIV infection are currently known. Characteristics which predict effective versus non-efficacious and which would further inform vaccine improvement therefore remain unclear (7). Investigation of possible mechanisms of vaccine-elicited immunity in pre-clinical and clinical trials and their subsequent influence on pathogenic HIV challenge is therefore needed to both uncover correlates of protection from HIV acquisition and to assist in the formulation of iteratively improved HIV vaccine candidates.

To date, the majority of HIV vaccine trials have utilized, either in part or completely, viral vectors (7). Viral vectors are viruses which are re-engineered to carry a given antigenic stimulus into the body and theoretically stimulate a host's immune system to respond more strongly against their carried antigen by virtue of the immune system's inherent ability to recognize virally-contained Pathogen-Associated Molecular Patterns (PAMPs). Viral vectors are attractive as vaccine candidates due to the relative ease and cost efficiency of producing them to high quantities compared to other vaccine modalities. Moreover, coupling an antigen of choice with viral PAMPs theoretically serves as a self-adjuvanting vaccine (14, 15). Due to these attractive properties, vaccine vectors are widely used as platforms for the elicitation of immune protection not just for HIV but also for infections such as Ebola, Rabies, and Canine distemper as well as therapeutic agents for several tumor types (16–21). Interestingly, it has been observed by several groups that the choice of viral vector for a given vaccine platform may have an influence on the quality of the adaptive immune response elicited (20, 22–24). It is therefore possible that viral vectors may not simply be passive shuttles, but play an active role beyond simple adjuvanting in influencing vaccine-elicited immune responses. As such, the identity of the virus utilized in vaccine construction and its biological properties may be relevant for understanding vaccine-elicited immune responses. While viral vectors have been constructed from broadly divergent viral families such as Flaviviruses, Alphaviruses, Poxviruses, Adenoviruses, Herpesviruses, Parvoviruses, and others, vectors based on Adenoviruses (Ad) and Poxviruses (Pox) have been most extensively utilized in the HIV vaccinology field (25). Importantly and as will be discussed below, these vectors have displayed pronounced differences in the magnitude and phenotype of adaptive immune responses they elicit. Furthermore, many of the Ad and Pox vectors used within HIV vaccine studies are replication-incompetent in human cells and several studies which have described their differential adaptive phenotypes used identical antigen transgenes. These points suggest that the nature of viral vector stimulus on the immune system at the time of vaccination may have an influential role on vaccine-

elicited immune responses. Therefore, a clear understanding of basic vector biology and its influence on vector-elicited innate and adaptive immune responses will be instrumental in understanding HIV vaccine-elicited immune responses and protection.

ADENOVIRAL VACCINE VECTORS – DEVELOPMENT AND CHARACTERISTICS

Fundamental Characteristics of Adenoviral Biology

Adenoviruses are a well-characterized viral family and have been utilized as model systems for many original discoveries of cellular biological processes such as transcription factor biology, RNA transcript splicing, and virus-host interactions (26–34). While Ad can differ in several basic biological properties relevant when considering them in their vectorized form, characteristics of their infectious life cycle are roughly similar. The genetic material of Ad is encoded on a linear double-stranded DNA genome encased within a non-enveloped icosahedral protein shell approximately 90 nm in diameter. This shell contains 11 structural proteins, which are responsible for mediating virus attachment to host cell receptors as well as viral entry into the cytoplasm (35). Ad initiates its infectious cycle by gaining entry into the cell by ligation of its fiber protein with its cognate cellular attachment receptor (36). The binding of the fiber protein then allows close association of RGD peptide motifs contained within the penton base proteins, which are found at the vertices of the viral capsid icosahedron, to associate with host cell α_v integrins (36–40). Ligation of α_v integrins signals for viral endocytosis via receptor-mediated endocytosis (40–44). Following endocytosis, Ad vectors lyse their containing endosome, and utilize host intracellular machinery to traffic along microtubules to the nuclear pore, where their genome is transferred into the host cell nucleus via a protein-complex association with cellular importins (45–50). As mentioned above, most Ad vectors follow these general life cycle parameters, but many variations on these basic biological parameters generate substantial diversity in Ad vectors, a wealth from which vaccinologists can select desirable strains upon which to base vaccine platforms.

Adenoviruses are a diverse group, infecting most known taxa of vertebrates and with currently 69 Human Ad known (51–56). These viruses are subcategorized into subgroups, or “Species”, classically based upon serotype cross-neutralization profiles which have been further supported by analysis of sequence homology. Importantly, Ad Species can differ in several key aspects of viral biology which may influence resultant innate and adaptive immune phenotypes when used as vaccine vectors. First, Ad vectors can utilize a variety of primary and secondary cellular attachment receptors to mediate cellular attachment and entry. As mentioned above, Ad use a two receptor system, with the distal knob domain of the Ad fiber protein binding with high affinity to its primary cellular attachment receptor. Several primary cellular attachment receptors have been identified or suggested for Ad vectors, such as Desmoglein 2, CD80/86, Heparin Sulfate Proteoglycans, or sialic acids, with the most thoroughly described being the tight junction component Coxsackievirus B and Adenovirus Receptor (CAR), or the complement receptor CD46 (57–68). Of these receptors, CAR has been shown to be the cellular attachment receptor for Species A, C, D, E, and F while CD46 has been shown to be utilized by the Species B and Species D Ad’s. These differences in primary receptor usage are important in that they influence Ad vector cellular tropism and therefore may influence the immune cell repertoire Ad vectors have access to or the target cell population which serves as the production site(s) for their transduced antigen (69–74).

A second way in which Ad vectors differ from one another are their intracellular trafficking patterns. Following their attachment to and endocytosis into their target cell populations, several Ad serotypes such as the Species C Ad’s Ad2 and Ad5 have been observed to enter their target cells and rapidly exit the endosomal space at the early endosome, with a half-life residence time in the endocytic pathway of approximately 3-15 minutes (75–78). Other Ads have been observed to be retained in the endocytic pathway for a much longer times, allowing them to gain access to the late endosomal compartment (79–83). This property has been observed with the Species B Ad’s Ad7 and Ad35 and

chimeric viruses containing their fiber proteins, but likely extends to other species B and D Adenoviruses as well. Interestingly, the differences outlined above in intracellular trafficking patterns correspond to primary cellular attachment receptor usage, with the Species C Ad's utilizing CAR and the Species B Ad's utilizing CD46 as their primary cellular attachment receptor. Furthermore, these differences in vector intracellular trafficking may influence subsequent Pattern Recognition Receptor (PRR) access as evidenced by differential abilities of Ad to stimulate TLR9, a late endosomal resident PRR (84). Differences between Ad vectors and their usage of cellular attachment receptors and endocytic trafficking patterns may therefore have a resultant influence on their ability to stimulate innate immune responses in a diverse array of immune cell subsets. These differences in Ad biological properties may therefore yield valuable insights into the mechanisms by which they stimulate innate and adaptive immune responses when utilized as vaccine vectors.

Derivation of Adenoviral Vectors

Unique aspects of Ad transcriptional regulation make them particularly amenable to vectorization in replication-incompetent formats. Following import into the nucleus, Ad genome transcription is initiated in a coordinated, program-wise manner. Ad genome length can range from 26–35kbp depending on the Ad serotype, and transcription from this dsDNA genome is controlled in two main phases. The first phase consists of the “early” proteins, which serve to hijack host cellular transcription factor machinery to facilitate viral transcription and to block host cell anti-viral defenses for augmented viral replication (85–87). This early phase ends with the induction of viral genome copying. The second phase consists of transcription mainly from the Major Late Promoter (MLP) from which a large number of the Ad structural proteins are coded by means of differential RNA splicing (88–91). Transcription through the early gene regions, in particular E1, is required by Ad vectors for full replication capacity and deletion of this region renders an Ad vector replication-incompetent except in cells which are able to provide E1 *in trans* (92–94). Deletion of the E1 and/or E3 region has the added

benefit of increasing the transgene coding capacity in Ad vectors to approximately 4,000 base pairs, enabling them to carry medium-sized protein transgenes (95). These properties, coupled with the ability of Ad to effectively transduce a broad array of tissues and their vastly reduced probability of malignant complications relative to lentiviral vectors, led to their initial development as gene therapy vectors (96).

Initial studies seeking to derive Ad vectors for usage in gene therapy applications focused predominantly on the Species C Ad5 (97). These vectors were used for transduction of several different tissues with genes supplementing genetic disorders such as cystic fibrosis and ornithine transcarbamylase deficiency as well as induction of angiogenesis through transduction of VEGF or FGF (98–101). Though initial studies utilizing these vectors were marred by cases of unacceptable levels of hepatotoxicity, these vectors remain widely used for many applications of gene therapy in several ongoing clinical trials (102). Furthermore, several groups have reported methods of Ad5 modification to circumvent possible hepatotoxicity such as receptor-mediated liver de-targeting, vector PEG-ylation, or Hexon protein HVR replacement with those from a non-serum factor-binding Ad (71, 103–107). While there have been difficulties in adapting Ad vectors for gene therapy applications, the ability of Adenoviruses to carry and express heterologous gene products, coupled with their ability to stimulate immune responses, have made them ideal platforms for further derivation for use as vaccines.

Ad vector utility and safety for vaccination by the intramuscular route has been extensively established by the low rate of adverse events observed in vaccination of thousands of military recruits with live Ad4 and Ad7 vaccines, as well as the many Phase I, II, and III clinical trials using Ad vectors which have shown high degree of vector tolerance and absence of severe adverse events (108–110). However, replication-competent unmodified Ad vectors can only carry approximately 2kb of external genetic information, limiting the available space for transgenes to single, small products (111). Replication-competent Ad vectors also carry with them the risk of replication and spread to immunocompromised individuals, such as those with clinical AIDS and cancer patients undergoing

chemotherapy. Generation of attenuated or replication-incompetent Ad vectors circumvented these concerns through deletion of the Ad E1, E3, and parts of the E4 protein segments. These “next generation” Ad vectors are able to carry multiple transgene products while also being unable to replicate except for in E1-complementing immortalized cell lines, increasing their safety for use in immunocompromised individuals and making them useful platforms for vaccine design (111). The ability of the expanded utility and safety of Ad vaccine vectors while also possessing a large transgene coding capacity, coupled with their relative lack of expense to produce at high quantities, has made them attractive platforms for vaccination against a variety of infections and cancers, and in particular as platforms for HIV vaccine design (112–115).

Adenoviral HIV Vaccines

Similar to efforts to derive Ad vectors for gene therapy, Initial efforts at HIV vaccine design utilizing Ad vectors again focused largely on Ad5-based vectors. In several pre-clinical and clinical studies, Ad5-based vectors were shown to be highly immunogenic relative to vaccine regimens utilizing DNA constructs or the poxviral vector Modified Vaccinia Ankara (MVA) either as a single injection or as part of a DNA-primed heterologous boost regimen. These measures of initial immunogenicity related to Ad5’s ability to induce potent IFN- γ ELISpot responses as well as protect vaccinated Rhesus monkeys from potent peak SHIV 69.6P or SIVmac239 viral loads and subsequent depletion of CD4⁺ T cells (116–119). The potent immunogenicity of Ad5-containing vaccine-regimens, coupled with their protection of rhesus monkeys from pathogenicity in an SIV challenge model led to the development of Ad5 in Phase I and Phase IIb proof of concept clinical trials. These studies, culminating in the STEP/Phambili trials, were unfortunately terminated prior to their planned endpoint for futility (120, 121). While disappointing due to the vaccine’s failure to provide protection and therefore immunological correlates of vaccine-elicited protection from infection, a more concerning observation from these studies was the trend toward increased HIV acquisition in vaccinated individuals who were Ad5 seropositive at the time of vaccination,

and in particular in uncircumcised men with high preexisting Ad5 antibody titers (122). The proposed mechanism of enhancement of HIV acquisition was postulated to occur via CD4⁺ memory T-cell reactivation and enhanced trafficking to the mucosa, where these cells would serve as an increased susceptible target cell reservoir for HIV infection. This “enhancement effect” has been actively investigated by several groups, yet several studies have reported that Ad5 seropositivity does not lead to marked induction of CD4⁺ T cell activation and trafficking upon vaccination (123–128). While this hypothetical effect remains an active concern for Ad5-based vaccines, a more important observation from this study was the possibility of pre-existing humoral immunity against an Ad5-based vector can potentially blunt elicited adaptive immune responses following vaccination rendering Ad5 a vector with limited clinical applicability as an HIV vaccine platform (122).

Concerns surrounding putative enhancement effect and reduced immunogenicity of Ad5 in the face of pre-existing humoral immunity motivated the derivation of immunogenic Ad vectors based on Adenoviruses against which a large proportion of the population does not have high levels of pre-existing humoral immunity. These studies have utilized a variety of strategies such as vectorization of non-human Adenoviruses such as those from Chimpanzee, Bovine, or other sources, “alternative serotype” human Adenoviruses vectors against which global seropositivity was less prevalent, or alteration of the Ad5 hexon hypervariable regions (HVR’s) to circumvent pre-existing humoral immune responses (107, 129–133). Vectors based on alternative serotype Species B and Species D Ad’s such as Ad35, Ad26, and Ad48 were found to elicit comparable immune responses relative to Ad5 in terms of antigen-specific systemic CD8⁺ T cell responses and IFN-γ ELISpot levels (133). Possible widespread utility of these vectors was shown by neutralization titers against these vectors in 200 volunteers in sub-Saharan Africa, as measured reciprocal serum neutralization titers of >1,000, was reported as 48% for Ad5 relative to 1%, 0%, and 0% for Ad35, Ad26, and Ad48, respectively. This result was further supported by a study of seroprevalence in 4,381 individuals from several geographic locations indicating

that high levels humoral immune responses against Ad5 were found in 14.2-43.2% of study populations, while those against Ad35, Ad26, and Ad48 were 0-1.0%, 0-2.5%, and 0-2.1%, respectively (134).

Importantly, these “alternative serotype” vectors were found to retain significant immunogenicity in the presence of pre-existing Ad5 immunity, highlighting their applicability as vaccine vectors for further pre-clinical development (22, 133, 135). Of note, several groups have reported conserved cross-reactive T cell epitopes present within the Adenovirus hexon genes of many serotypes indicating that Ad serostatus may not be the only immunological influence on Ad vector cross-reactivity following vaccination (136–138). Whether this pre-existing T-cell cross-reactivity in Ad vectors also influences Ad vector-elicited vaccine responses remains to be described.

The observation that Ad vectors based on alternative serotypes were immunogenic and stable prompted their further characterization as possible vaccine platforms. Interestingly, initial characterization of alternative serotype Ad's indicated that, while many of these constructs resembled Ad5 in terms of the magnitude of their elicited cytotoxic T cell responses and levels of induced neutralizing antibodies, several alternative serotype human Ad's induced phenotypically distinct T cell responses comparatively (22, 131, 139). In particular rhesus monkeys boosted Ad35 or Ad26 following and Ad5 prime were found to elicit a greater breadth of anti-SIV Gag protein IFN γ ELISpot responses, greater magnitudes of anti-Gag antibodies, and more polyfunctional T lymphocyte responses relative to homologous prime-boost of animals with an Ad5 vector. These observations are especially important given that T-cell responses targeted to the Gag protein and T-cell polyfunctionality have previously been described as correlated with lowered pathogenicity during HIV/SIV infections (140–142). The importance of these immune responses was further shown by vaccination with Ad35- and Ad26-based vectors conferring increased protection against CD4⁺ T cell depletion and lowered set-point viral loads in SIV-infected rhesus monkeys relative to Ad5/Ad5 homologous prime-boost vaccination. These results suggest that while Ad5 and alternative serotype Ad vectors may elicit cytotoxic T cell and antibody

responses of similar magnitudes, their phenotypes may differ markedly, having a pronounced influence on disease acquisition and progression. Understanding the source of these differences is therefore crucially important for the further optimization and construction of novel Ad vectors with the goal of improving protection against HIV acquisition and disease. Of note, the vectors utilized in the protective study above were deleted for their E1 and E3 genes, rendering them replication incompetent. Furthermore, they carried identical transgene products. This suggests that the protective properties of Ad35- and Ad26-containing regimens may be in part due to differences in vector biology of Ad35 and Ad26 relative to Ad5, potentially due to induction of differential innate immune milieus following vaccination, leading to differences in formation of resultant vaccine-elicited adaptive immune phenotypes.

The increased efficacy against SIV infection and pathogenesis of Ad26 vaccine regimens in the Rhesus monkey pre-clinical model has led to their further development for pre-clinical and Phase I clinical studies of immunogenicity in humans, either alone or in combination with Poxviral vectors or proteins and continues to yield exciting and novel insights into the methods by which HIV acquisition and pathogenesis can be prevented (143–145). Further studies to characterize and probe the mechanisms by which alternative serotype Ad vectors are able to elicit distinct immune responses relative to Ad5, and the influence of viral vector biological properties on these responses will yield crucial insights into vaccine design not only for HIV but for a variety of other pathogens and cancers as well.

POXVIRAL VECTOR DERIVATION AND USAGE IN HIV VACCINE TRIALS

Smallpox, caused by the Variola major and Variola minor viruses and which killed the most individuals of any infectious disease in recorded human history, was confirmed eradicated on the globe by the World Health Organization in 1979 (146–148). This was due in large part to the usage of

prophylactic vaccination against Smallpox using Vaccinia virus. Vaccination with Vaccinia virus, though highly effective at preventing infection with one of the two viral agents of Smallpox, in the absence of the risk of Smallpox infection also carries an unacceptable risk of complications such as fever, rash, progressive Vaccinia infection, and eczema vaccinatum (149). Due to this undesirably high rate of possible complications following Vaccinia virus vaccination global vaccination with Vaccinia has been halted. However the possible emergence of novel orthopoxvirus pathogens such as Monkeypox as well as amenable genetic and molecular characteristics of the poxvirus family, such as the ability to express large transgene inserts under native poxviral protein transcriptional levels and comparably high virion stability, have made poxvirus vectors attractive platforms for the generation of virally-vectored vaccines against a variety of pathogens (21, 150).

The risk of adverse reactions to Vaccinia virus-vaccination and the possibility of pre-existing immunity against Vaccinia virus in a large, albeit diminishing, proportion of the population with immunity to the virus blunting vectored immune responses have led to the generation of several Poxviral vectors which were further attenuated or antigenically-distinct relative to Vaccinia (151). One such derived vector is Modified Vaccinia Ankara (MVA). MVA is derived from the Chorioallantoid Vaccinia Ankara vaccine strain of Vaccinia Virus, and was attenuated by serial passage in chicken embryo fibroblasts 570 times (152). This process generated a virus strain which has lost approximately 30% of its genome, much of it in six regions containing genes commonly referred to as host-range genes such as intracellular signaling cascade antagonists and decoy cytokine receptors which serve the purpose of blunting host immune responses against viral infection (153). Importantly, attenuation of MVA through serial passage rendered it unable to replicate in most mammalian cell types as well as replication incompetent in human tissues while retaining the ability to express exogenous proteins to high levels (154–156). A second, more targeted approach was taken by another laboratory which specifically deleted 18 Open Reading Frame's (ORF) from the Copenhagen strain of Vaccinia virus, creating the New

York Vaccinia Virus, or NYVAC (157). Although this vector retains much of the host range genes originally contained in its parental Copenhagen strain, it displays a vastly attenuated phenotype similar to that of MVA, being unable to complete replication in human tissues (158). A third strategy aiming to utilize a poxvirus vector to stimulate potent immune responses while circumventing pre-existing Vaccinia virus immunity Avipoxviruses, which are poxviruses which cause disease in several species of birds. Of these, the Canarypox-virus based vector ALVAC widely used in vaccinology (159). Avipoxvirus vectors such as ALVAC and Fowlpox-based vectors are extremely attenuated in mammals and are unable to replicate in mammalian cells (160).

As mentioned above, poxviruses contain an arsenal of host range or “security” proteins which serve to block host immune responses against viral infection via antagonism of innate immune viral sensing, cytokine signaling interference, downregulation of antigen presentation, as well as other mechanisms (161–165). Deletion or host-virus mismatch of these proteins renders the virus more vulnerable to immune attack, leading to a more attenuated viral phenotype. Genetic sequencing and comparison of the poxviral vectors MVA and NYVAC has shown that MVA, despite having lost approximately 30% of its original genome, retains some of its ORF’s which antagonize pro-inflammatory responses while NYVAC retains many of its proteins which counteract interferon-signaling (153, 157). Interestingly, several studies have indicated that this difference in security protein arsenals was made apparent in immune gene transcriptional responses in MVA or NYVAC infected HeLa and dendritic cell populations, with MVA inducing a greater interferon-related transcriptional response than NYVAC, and NYVAC inducing a more proinflammatory response relative to MVA responses which matched the previously-observed deletion in security protein arsenals in these vectors (166–168). The importance of Poxviral security protein arsenals in viral attenuation was further shown when modification of MVA or NYVAC for further attenuation by removal of additional security proteins increased their immunogenicity in several in vitro and in vivo systems (24, 169–171). These observations together

suggest a large role for poxviral security protein arsenals in the influencing of poxvirus vector-elicited innate immune responses. While similar characterization of the Avipoxvirus ALVAC security protein arsenal has not been performed, it is important to note that, as mentioned above, ALVAC is unable to replicate in mammalian cells. This suggests that while ALVAC's security protein arsenal remains genetically intact, mismatch between these proteins and mammalian host targets may render them functionally inert. This hypothesis is further supported by the observation that ALVAC is able to induce several cytokines in vivo in response to vaccination, indicating that it is unable to at least control host innate immune sensing of it (160). Together, these studies suggest that while Pox vectors may be structurally similar they may be immunologically distinct from one another, likely depending on the degree and identity of security protein ORF's which have been deleted and/or mismatched relative to their host.

Similar to Ad vectors, the outstanding safety profile of the attenuated Poxviral vectors MVA, NYVAC, and ALVAC has led to their extensive usage in HIV vaccine design. All three vectors have been utilized extensively in pre-clinical clinical models in which they as have shown a pronounced ability to elicit polyfunctional CD4⁺ T cell responses as well as robust antibody titers against their carried transgene, both in isolation in addition to as part of heterologous prime-boost regimens. These studies are extensively reviewed elsewhere (7, 21, 172, 173). Of note however is the RV144 "Thai trial." In this trial approximately 16,000 individuals in Thailand received an ALVAC-based vector prime followed by sequential boosting with HIV Env gp120 recombinant protein (174). This study is currently the only HIV vaccine trial in which a putative protective effect on HIV acquisition was seen, with an observed 31.2% protection from HIV acquisition in vaccinees. While it is a major breakthrough to the field of HIV vaccinology that this trial showed lowered risk of acquisition, it is also important to note that those individuals who were infected displayed no control of HIV viral replication, with vaccinated and sham-vaccinated individuals showing no difference in peak or setpoint viral loads. As will be discussed below,

this study was also the first in which correlates vaccine-elicited immune responses on risk of HIV infection were observed in a clinical trial in humans (175). In light of this observation, it becomes increasingly important to understand the mechanisms by which adaptive immune responses are elicited by vaccine modalities. As with Ad vectors, the pronounced differences in pox vector biology outlined above may influence resultant immune responses against their carried transgenes. It remains to be determined however, whether the differences in security protein arsenals of ALVAC, MVA, and NYVAC play a role in initial immune activation and subsequent adaptive immune phenotype stimulation. Understanding their phenotypes and function in the elicitation of vaccine-elicited immune responses is therefore critical for the further improvement of pox vectors for HIV vaccine development.

HIV VACCINE TRIALS AND THE SEARCH FOR PROTECTIVE CORRELATES

While many promising vaccine modalities are currently in development, HIV vaccine design to date has been unable to produce a vaccine of sufficient efficacy for global application (178,179). As mentioned above, factors which serve to make the search for an effective HIV vaccine difficult include extensive viral global diversity, the rapidity with which a virus can evade immune pressure owing to an extremely fast mutation rate, and the likely requirement of mucosal immune responses much stronger or of a different phenotype than those that are induced with previously-developed vaccine modalities (178–181). This search is further hampered by the point that correlates of protection from HIV acquisition are still not known (184,185). In contrast to many other viral pathogens, exposure to and infection with HIV does not confer immunity against subsequent exposures, as evident by common occurrences of HIV super-infection with secondary strains (184). However, despite the common occurrence of super-infection with HIV, immune responses which are able to protect against HIV infection or pathogenesis can be elicited during natural infection. For example, both in pre-clinical and clinical settings, breadth and magnitude of cytotoxic CD8⁺ T cell responses against the HIV Gag protein

were found to influence viral control (143, 185, 186). Additionally, genetic factors such as MHC and KIR haplotype have been found to influence infection trajectory (187–189). Antibodies have also been observed by several groups to arise late in infection and which can neutralize a large proportion of HIV global isolates (190–192). Cellular and humoral immune responses which can protect against HIV acquisition and pathogenesis therefore are able to be generated by the human immune system. A major focus of HIV vaccine research centers on how these rare responses can be specifically generated or enhanced with a vaccine.

To date, over 60 pre-clinical and clinical studies have been performed with the goal of developing an HIV vaccine (7, 8). Vaccine preparations have utilized a wide variety of components such as recombinant HIV protein formulations, viral vectors containing segments of HIV proteins, cladal or global consensus sequence recombinant proteins, as well as mosaic vector transgenes aimed at best covering global T cell epitope diversity. While many vaccine modalities have been tested in the Rhesus monkey SIV infection preclinical model, to date only four have progressed to large scale Phase IIb/III clinical trials in humans with three showing futility for efficacy. The first of these studies focused on utilizing recombinant proteins meant to elicit HIV neutralizing antibodies through vaccination with either a Clade B or a Circulating Recombinant Form (CRF) AE antigen, and are commonly known as the VAX003/004 studies (9, 193). The second set of studies, as mentioned above, were the Step/Phambili trials, in which DNA and Ad5 prime/boost regimens targeted the HIV Gag, Pol, and Nef proteins of Clade B (121). The most recent vaccine trial conducted, known as the HVTN 505 study, consisted of a DNA prime, Ad5 boost immunogen set expressing HIV Gag, Pol, and Env consensus sequences from clades A, B, and C. Similar to the STEP/Phambili trials, this study was recently halted due to futility (194). Notably, these studies were found to elicit their targeted vaccine immune responses, namely anti-immunogen antibody responses in the case of VAX studies, and cytotoxic CD8⁺ T cell responses in the STEP/PHAMBILI and HVTN505 studies. However, the lack of efficacy of these responses elicited by these vaccines

suggests that classically-defined immune responses elicited by vaccine modalities previously used for other diseases may be insufficient to confer protection against HIV infection, and therefore mechanisms of alternative immune response elicitation will be important for understanding future vaccine trial successes.

The fourth large-scale HIV vaccine trial was the above-mentioned RV144 “Thai trial”. In this study, vaccinees were injected with an ALVAC vector expressing the CRF-AE Gag, Pol, and Env genes and subsequently boosted with HIV gp120 B/E recombinant protein. As mentioned above, this study was the first to observe a reduction in HIV acquisition risk in vaccinees which had a 31.2% lowered chance of acquisition through 3.5 years following vaccination (174). Importantly, correlates of risk analysis on this study indicated for the first time two particular immunological correlates which may have had an influence on HIV acquisition risk (175). This clinical trial is of special importance for two reasons. The first is that it demonstrated for the first time that it is possible to elicit immune responses in a large human population which lower the risk of HIV acquisition. Secondly, it provided two correlates of lowered risk for HIV acquisition, giving researchers clues as to the possible means by which protection against HIV infection might be conferred by a vaccine. The first finding was that IgG binding, but not neutralizing, antibodies to the Env V1/V2 region was negatively associated with HIV acquisition. The second correlate observed was that IgA binding antibodies to HIV Env were positively associated with HIV acquisition. These findings are interesting given that antibody recognition but not neutralization of HIV suggests previously unknown effector mechanisms which may be relevant and important for protection from HIV infection. Interestingly, the correlates of lowered risk in the RV144 trial are unique from those suggested by the pre-clinical model, mainly breadth and magnitude of Gag-specific T cell responses and neutralizing antibody titers. This suggests that there may be several different mechanisms by which a vaccine could confer protection against HIV infection, and that combination of these different mechanisms may synergize for increased protection.

While the RV144 immunological analysis indicates the importance of including an Env immunogen in vaccine formulations for the prevention of acquisition, it still does not inform methods of HIV virological control, suppression, or clearance. Furthermore, many of the immunological measurements for the determination of correlates of protection focus on adaptive immune phenotypes generated weeks or months following vaccination, and many focus on classically defined parameters of vaccines which have previously been observed to not confer significant protection against HIV/SIV. As suggested above, it is possible that there are several different means by which protection from HIV can be induced. In the over 60 clinical trials done to date, many have utilized Ad and/or Pox vectors, and these vectors continue to be used extensively in pre-clinical and clinical development (7). However, despite their widespread usage, the means by which immune responses, and in particular innate immune responses, elicited by these biologically distinct vectors influence resultant adaptive immune responses remains poorly characterized. The importance of innate immune responses in shaping adaptive immune phenotypes through cell-cell interactions and cytokine communication has been previously shown (195–198). Therefore, putative differential stimulation of innate immune responses by Ad and Pox vectors and the means by which it shapes vaccine-elicited immune responses may be of critical importance for understanding and putting into immunological context protective vaccine responses elicited in future HIV vaccine trials. Furthermore, using these vectors as a model system to understand the means by which adaptive immune phenotypes are established will yield important insight into future rational immunogen and vector design.

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Chapter 2: Vaccination with Adenovirus Serotypes 35, 26, and 48 Elicits Greater Innate Cytokine Responses Than Adenovirus Serotype 5 in Rhesus Monkeys

This chapter is based on the peer-reviewed publication:

Teigler, J.E., Iampietro, M.J., and Barouch, D.H. 2012. Vaccination with adenovirus serotypes 35, 26 and 48 elicits higher levels of innate cytokine responses than adenovirus serotype 5 in rhesus monkeys. *Journal of Virology*. **86**: 9590-9598.

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ABSTRACT

Adenovirus (Ad) vaccine vectors have proven highly immunogenic in multiple experimental models, but the innate immune responses induced by these vectors remain poorly characterized. Here we report innate cytokine responses to 5 different Ad vectors in 26 rhesus monkeys. Vaccination with Ad35, Ad26, and Ad48 induced substantially higher levels of anti-viral (IFN- γ , IP-10) and proinflammatory (IL-1RA, IL-6) cytokines as compared with Ad5 on day 1 following immunization. *In vitro* studies with capsid chimeric vectors and receptor blocking monoclonal antibodies suggested that fiber-receptor interactions as well as other capsid components were critical for triggering these innate responses. Moreover, the innate immune profiles likely involved multiple cell populations, including dendritic cells, monocytes/macrophages, and T lymphocytes. These data demonstrate that Ad35, Ad26, and Ad48 that utilize CD46 as their primary receptor induce significantly greater innate cytokine responses than Ad5, which uses the coxsackievirus and adenovirus receptor (CAR). These differences in innate triggering result in markedly different immunologic milieus for the subsequent development of adaptive immune responses by these vaccine vectors.

INTRODUCTION

Adenovirus (Ad) vectors are widely used for vaccination due to their immunogenicity, relatively large transgene coding capacity, and multiple available serotypes with diverse biological properties. While considerable data have been generated regarding adaptive immune responses elicited by Ad vectors, much less is known about innate immune responses induced by these vectors. As innate immune induction is critical for understanding both reactogenicity and adaptive immunity, it is important to define the innate pathways triggered by Ad vectors from various serotypes. Accumulating evidence suggests that different serotype Ad vectors induce qualitatively different adaptive immune response phenotypes (1–4). In particular, vaccine studies using the simian immunodeficiency virus (SIV) infection model in rhesus monkeys have shown qualitative differences in immune responses elicited by various serotype Ad vectors (3), which translated into different levels of protective efficacy against SIV challenges (5–7). However, innate immune profiles of different alternative serotype Ad vectors have not previously been studied in nonhuman primates.

Adenoviruses are a diverse group of double stranded DNA viruses with at least 65 known human serotypes, which are subdivided into species A-G based upon sequence homology (8–11). Vectors constructed using these viruses have been shown to differ significantly in terms of primary receptor usage (1, 12–15), intracellular trafficking patterns (16–19), transduction and activation of dendritic cells (20–25), utilization of secondary receptors (26, 27), cellular tropism (28–33), and interaction with pattern recognition receptors (PRR) (34–36). Notably, Ad5 utilizes CAR as its primary cellular receptor, whereas Ad35, Ad26, and Ad48 utilize CD46 (37). The species C adenovirus 5 (Ad5), the species B2 Ad35, and the species D Ad26 are currently being evaluated as vaccine candidates in clinical trials, yet relatively little is known about the possible differences in innate immunity induced by these vectors.

In this study, we describe the innate cytokine profiles induced in vivo by Ad vectors from 5 serotypes in 26 rhesus monkeys. We then assessed the mechanism of differential viral triggering of these innate responses using capsid chimeric vectors and receptor blocking monoclonal antibodies in vitro in human PBMC. Our studies demonstrate that Ad35, Ad26, and Ad48 vectors that utilize CD46 as their primary cellular receptor trigger profoundly different innate cytokine profiles characterized by higher levels of anti-viral and proinflammatory cytokines and chemokines compared with Ad5 vectors that utilize CAR.

RESULTS

Ad35, Ad26, and Ad48 induce more potent anti-viral and proinflammatory cytokines and chemokines compared with Ad5 following vaccination of rhesus monkeys. We initiated studies by assessing serum cytokine levels in rhesus monkeys following vaccination with 5 different serotype Ad vectors in 26 rhesus monkeys. Rhesus monkeys (n=4-8/group) were immunized i.m. with 3×10^{10} vp Ad5, Ad35, Ad26, Ad48, or chimeric Ad5 with the hexon hypervariable regions (HVR) replaced with those of Ad48 (Ad5HVR48) expressing SIV Env/Gag/Pol (38)(1). All vectors were replication-incompetent E1/E3-deleted Ad vectors that were prepared similarly and exhibited comparable characteristics, specific infectivity, and purity (1). Sera were collected on days 0, 1, 3, 7, 10, 14 and 28 following vaccination, and cytokine levels were assessed by luminex assays.

Longitudinal analysis of cytokine responses following vaccination with Ad5 revealed only low levels of anti-viral and proinflammatory cytokine and chemokine induction on day 1 following vaccination (Figures 2.1A & 2.1B). In contrast, animals vaccinated with Ad35, Ad26, and Ad48 displayed pronounced induction of multiple cytokines and chemokines on day 1 following vaccination. Increased induction by Ad35, Ad26, and Ad48 as compared with Ad5 was observed for the inflammatory markers IL-1RA (17.7-, 8.2-, 26.2-fold greater induction than Ad5, respectively; $p=0.006$, $p=0.009$, $p=0.02$, respectively, Mann-Whitney U Test) and IL-6 (8.3-, 4.0-, and 3.4-fold greater induction than Ad5, respectively; $p=NS$, $p=0.04$, and $p=0.03$, respectively). Additionally, significant induction of IFN- γ (1.4-, 1.7-, and 1.5-fold greater induction than Ad5; $p=NS$, $p=NS$, $p=0.02$, respectively), and its downstream-signaled chemokines IP-10 (8.3-, 6.0-, and 4.9-fold greater induction than Ad5; $p=0.03$, $p=0.009$, $p=0.03$, respectively) and I-TAC (2.8-, 1.1-, and 3.6-fold greater induction than Ad5; $p=0.03$, $p=NS$, $p=0.03$, respectively) was observed in animals that received Ad35, Ad26, or Ad48. These data demonstrate that

Ad35, Ad26, and Ad48 trigger substantially greater anti-viral and proinflammatory cytokine responses than Ad5 following vaccination.

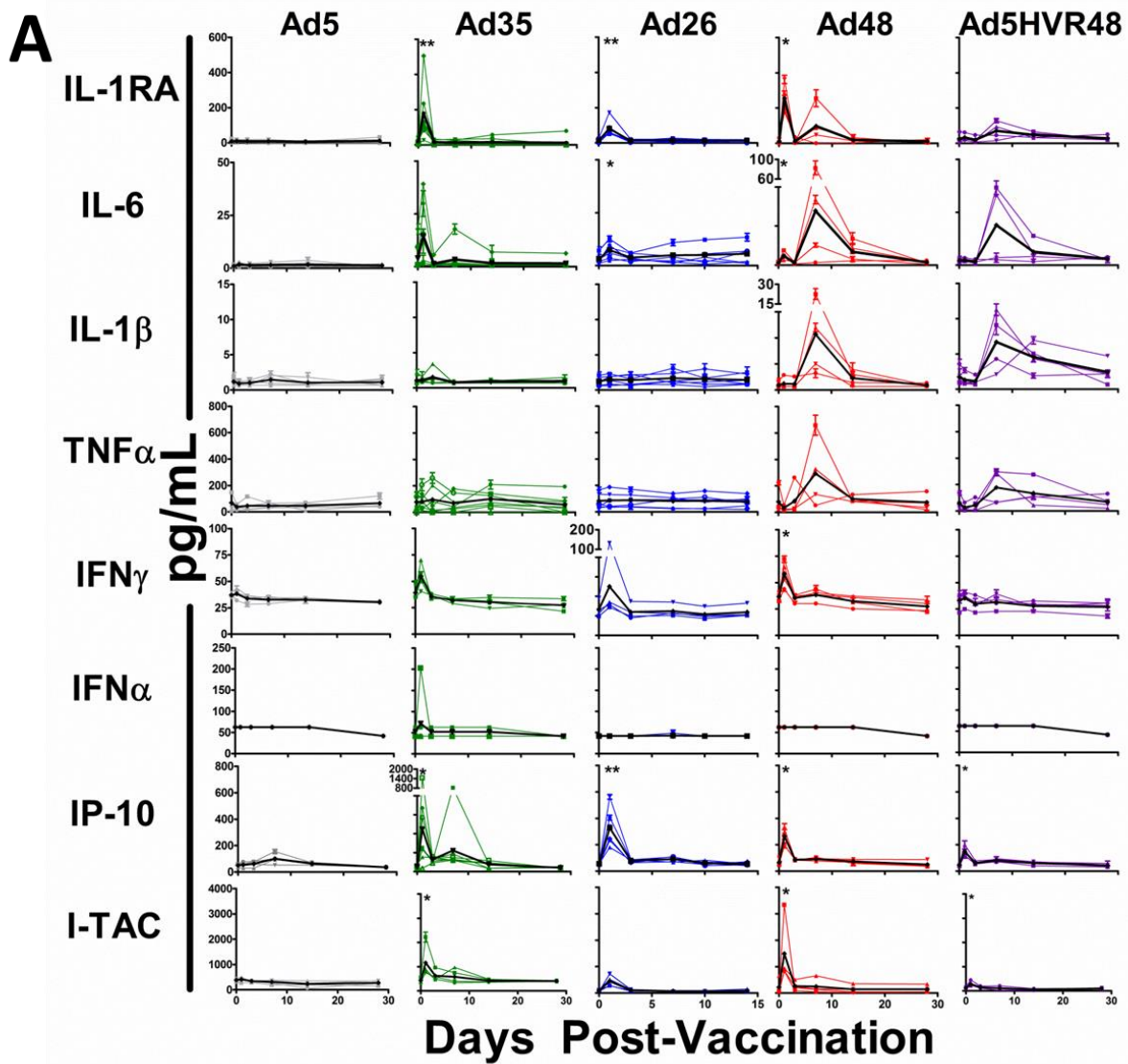


Figure 2.1. Serum concentrations of cytokines and chemokines in rhesus monkeys following vaccination with Ad vectors. Rhesus monkeys ($n=4-8/\text{group}$) were injected IM with 3×10^{10} vp Ad5, Ad35, Ad26, Ad48 or Ad5HVR48 vectors. Sera were collected on days 0, 1, 3, 7, 10, 14 and 28 following vaccination, and systemic levels of cytokines and chemokines were measured by luminex and ELISA assays. (A) Systemic levels of selected cytokines are shown in colored lines with means in black lines. Groups are compared by fold changes over baseline compared with Ad5 * $p<0.05$, ** $p<0.01$.

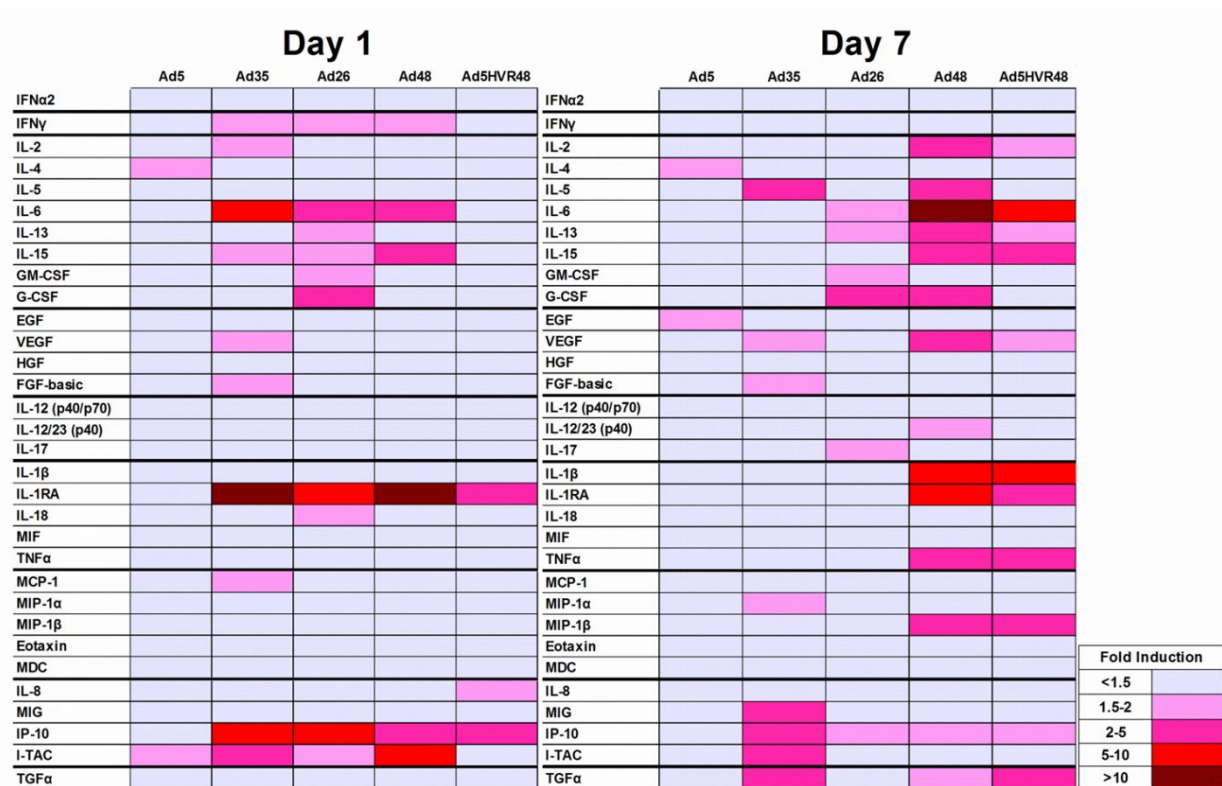


Figure 2.1., continued. (B) Mean fold induction of all cytokine responses relative to baseline by the various Ad vectors in rhesus monkeys one day (left) and seven days (right) following immunization with the indicated adenoviral vector. Data are represented as fold change over averaged baseline.

Interestingly, on day 7 following vaccination, animals that received Ad48 or the chimeric Ad5HVR48 vectors also displayed a temporally distinct peak of cytokine induction characterized by the proinflammatory cytokines IL-1RA, IL-6, IL-1 β and TNF- α (Figures 2.1A & 2.1B). This cytokine peak was not observed with Ad5, Ad35, or Ad26 vectors. Since Ad48 and Ad5HVR48 only share common hexon HVR sequences, we speculate that this day 7 cytokine peak may be triggered by hexon rather than fiber-receptor interactions. These data suggest that both fiber and hexon may contribute to triggering innate immune responses.

Cytokine induction following heterologous boost immunization. We next assessed whether innate cytokine responses following an Ad26 boost immunization would be impacted by previous priming with a heterologous Ad vector. Systemic cytokine levels were assessed in rhesus monkeys (n=4/group) primed with Ad35, Ad48, or Ad5HVR48 vectors as described above and then boosted 24 weeks later i.m. with 3×10^{10} vp Ad26 expressing SIV Env/Gag/Pol. Sera were collected and analyzed as described above. Longitudinal analysis of animals revealed cytokine profiles on day 1 following the boost immunization (Figure 2.2) that appeared similar to the cytokine profiles induced following priming with Ad26 (Figure 2.1A), including IFN- γ , IL-6, IP-10, and IL-1RA (Figure 2.2). These data suggest that the innate cytokine profiles following a boost immunization are dictated primarily by the vector utilized for the boost and are not substantially imprinted by the heterologous vector utilized for priming.

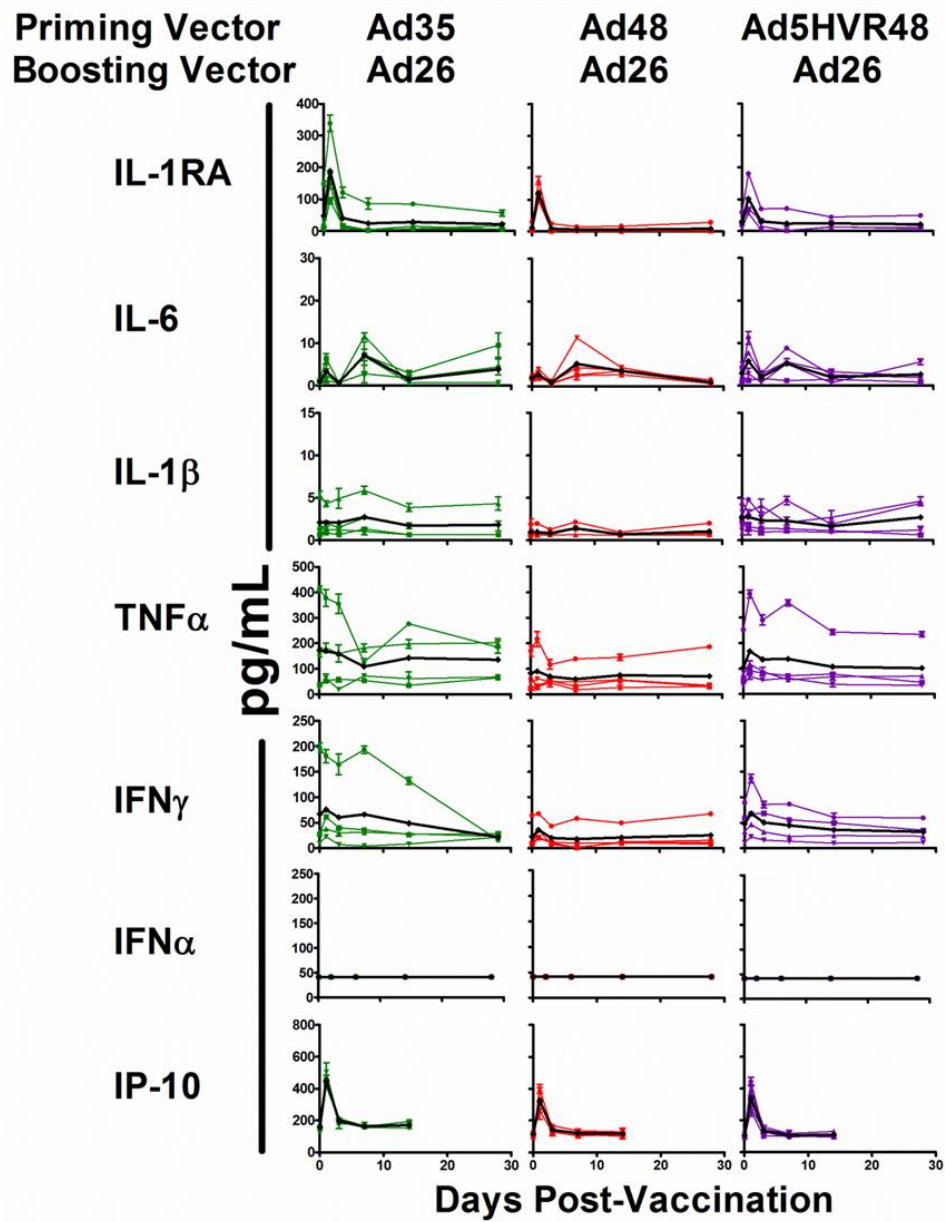


Figure 2.2. Serum concentrations of cytokines and chemokines in rhesus monkeys following boost vaccination with Ad26 vector. Rhesus monkeys ($n=4/\text{group}$) were boosted IM with 3×10^{10} vp Ad26 vector 24 weeks after priming with 3×10^{10} vp of Ad35, Ad48, or Ad5HVR48 vectors. Sera were collected on days 0, 1, 3, 7, 14, and 28 following vaccination and systemic levels of cytokines and chemokines were measured by luminex assays. Systemic levels of selected cytokines are shown in colored lines with group means in black lines.

Ad35, Ad26, and Ad48 induce higher levels of anti-viral and proinflammatory cytokines and chemokines compared with Ad5 in vitro. We next sought to probe the mechanism by which Ad vectors elicit differential innate immune cytokine profiles. We therefore assessed the capacity of Ad5, Ad35, Ad26, and Ad48 to trigger secretion of innate cytokines in vitro in freshly isolated human PBMC. Human PBMC (n=8-13/group) were stimulated with Ad5, Ad35, Ad26, or Ad48 at a multiplicity of infection (MOI) of 10^3 viral particles (vp) per cell. Cytokine and chemokine responses were measured in culture supernatant 24 hours post-infection by luminex assays.

Compared with the CAR-utilizing vector Ad5, the CD46-utilizing vectors Ad35 and Ad26 induced substantially higher levels of IFN- α 2 (128- and 96-fold higher levels, $p < 0.001$ and $p < 0.01$, respectively, Kruskal-Wallis test with Dunn's correction for multiple comparisons), IFN- γ (38- and 24-fold higher levels, respectively, $p < 0.001$), IL-1 β (10- and 5-fold higher levels, $p < 0.001$ and $p < 0.01$, respectively), and TNF- α (10- and 4-fold higher levels, $p < 0.001$ and $p < 0.01$, respectively) (Figure 3A). Ad35 and Ad26 also induced higher levels of MIP-1 α (18- and 8-fold, respectively, $p < 0.001$), MIP-1 β (16- and 9-fold, respectively, $p < 0.001$), IL-6 (2.4- and 1.4-fold higher levels, $p < 0.001$ and $p = \text{NS}$, respectively) and IL-1RA (3- and 3-fold higher levels, respectively, $p < 0.001$) (Figure 2.3A). The species D vector Ad48 displayed an intermediate phenotype of cytokine and chemokine induction relative to Ad5 and those induced by Ad35 and Ad26. A complete analysis of all cytokines and chemokines measured in the luminex assays demonstrated that Ad35, Ad26, and Ad48 induced higher levels of multiple anti-viral and proinflammatory cytokines, as well as chemokines including MCP-1 and IP-10 as compared with Ad5 (Figure 2.3B). These in vitro data are largely consistent with the in vivo data from vaccinated rhesus monkeys with a few notable exceptions, such as the high levels of IFN- α 2 induced in vitro compared with in vivo.

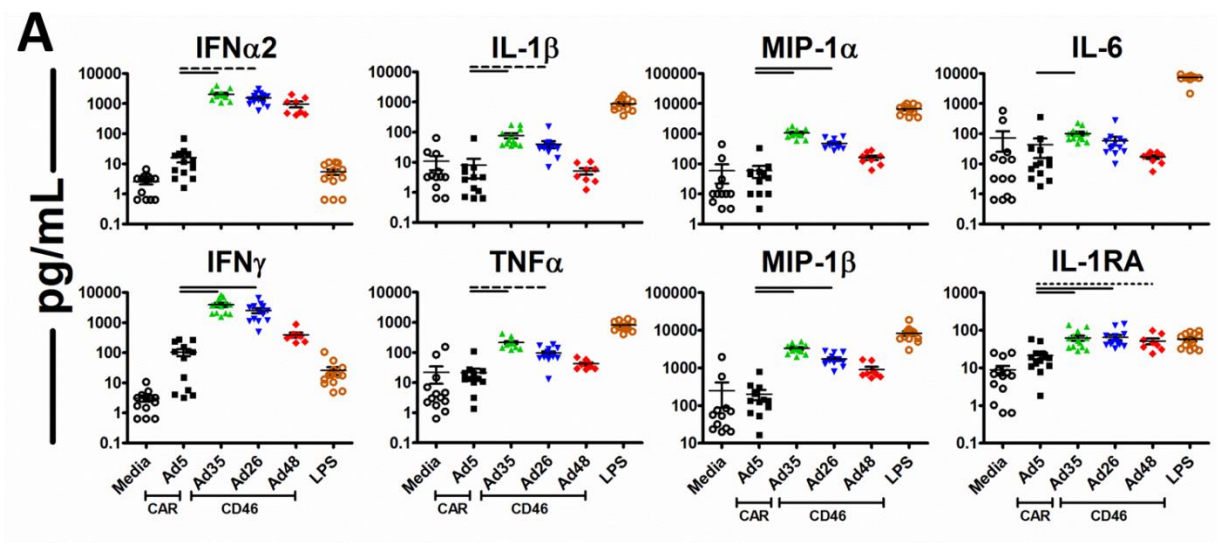


Figure 2.3. Cytokine and chemokine responses induced *in vitro* in human PBMC following Ad vector stimulation. (A) Individual PBMC ($n=8-13/\text{group}$) were stimulated with 10^3 vp/cell of Ad5, Ad35, Ad26, or Ad48 vectors and cytokine responses measured after 24h by luminex assays. LPS (1 ng/mL) was used as the positive control. Data are represented as individual measurements with mean responses indicated by a solid line \pm SEM. Bars indicate $p < 0.05$ (dotted line), $p < 0.01$ (dashed line), and $p < 0.001$ (solid line) using Kruskal-Wallis tests with Dunn's correction for multiple comparisons.

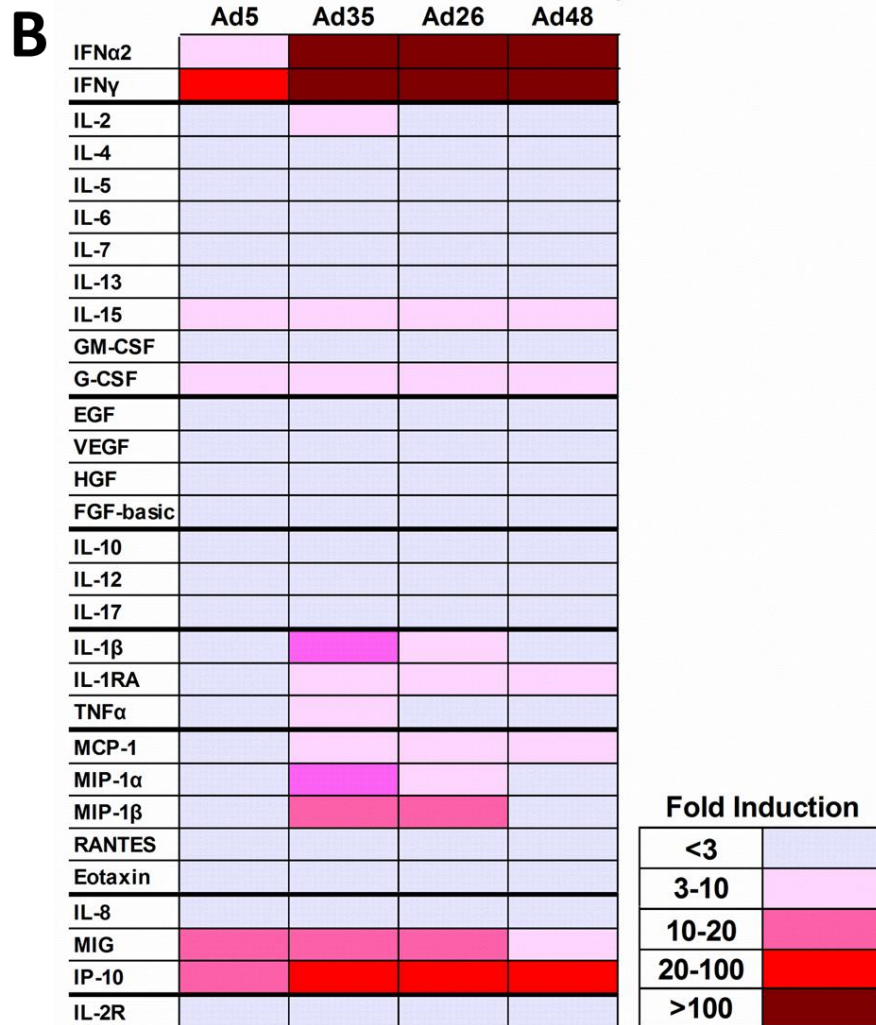


Figure 2.3., continued. (B) Mean group fold induction of all cytokine responses relative to media by various Ad vectors in human PBMC.

Both fiber and capsid components contribute to innate immune stimulation by Ad vectors. We next explored the role of the key structural proteins of the adenovirus capsid on innate immune stimulation. We utilized a panel of chimeric Ad5/Ad35 (39) vectors and stimulated human PBMC (n=8/group) as described above. Chimeric Ad vectors included Ad5 with the Ad35 fiber (Ad5f35) or Ad35 fiber and penton (Ad5p35f35), and Ad35 with the Ad5 fiber (Ad35f5) or Ad5 fiber knob (Ad35k5). Replacement of the Ad5 fiber with the Ad35 fiber, or Ad35 fiber and penton, resulted in an increased induction of multiple cytokines relative to Ad5, including IFN- α 2 (126- and 184-fold higher levels, $p<0.01$ and $p<0.001$, respectively), IFN- γ (16- and 9-fold higher levels, $p<0.05$ and $p<0.01$, respectively), IL-1 β (2.5- and 1.6-fold higher levels, respectively $p=NS$), TNF- α (12- and 11-fold higher levels, respectively, $p<0.001$), MIP-1 α (52- and 40-fold higher levels, $p<0.01$ and $p<0.001$, respectively), MIP-1 β (30- and 25-fold higher levels, respectively, $p<0.001$), IL-6 (7- and 7-fold higher levels, respectively, $p<0.001$) and IL-1RA (4.4- and 3.5-fold higher levels, $p<0.05$ and $p<0.01$, respectively) (Figure 2.4).

A corresponding decrease in stimulatory capacity was observed with Ad35 vectors containing the Ad5 fiber or fiber knob as compared to Ad35, suggesting the importance of the Ad35 fiber for innate cytokine stimulation. For example, Ad35f5 and Ad35k5 induced lower levels relative to Ad35 of IFN- γ (4.7- and 7.1-fold lower levels, $p<0.05$ and $p<0.01$, respectively), IL-1 β (2.7- and 10.6-fold lower levels, $p<0.05$ and $p<0.001$, respectively), TNF- α (5.5- and 7.1-fold lower levels, respectively, $p<0.01$), MIP-1 α (13.0- and 23.6-fold lower levels, $p<0.01$ and $p<0.001$, respectively), MIP-1 β (6.0- and 9.4-fold lower levels, respectively, $p<0.05$) and IL-6 (3.4- and 5.2-fold lower levels, $p<0.05$ and $p<0.01$, respectively) (Figure 2.4). However Ad35f5 and Ad35k5 still induced most cytokines to a greater level than did Ad5, indicating that other capsid components in addition to fiber also likely contribute to innate cytokine stimulation. Taken together, these results suggest that both fiber and other capsid components such as hexon influence innate immune induction.

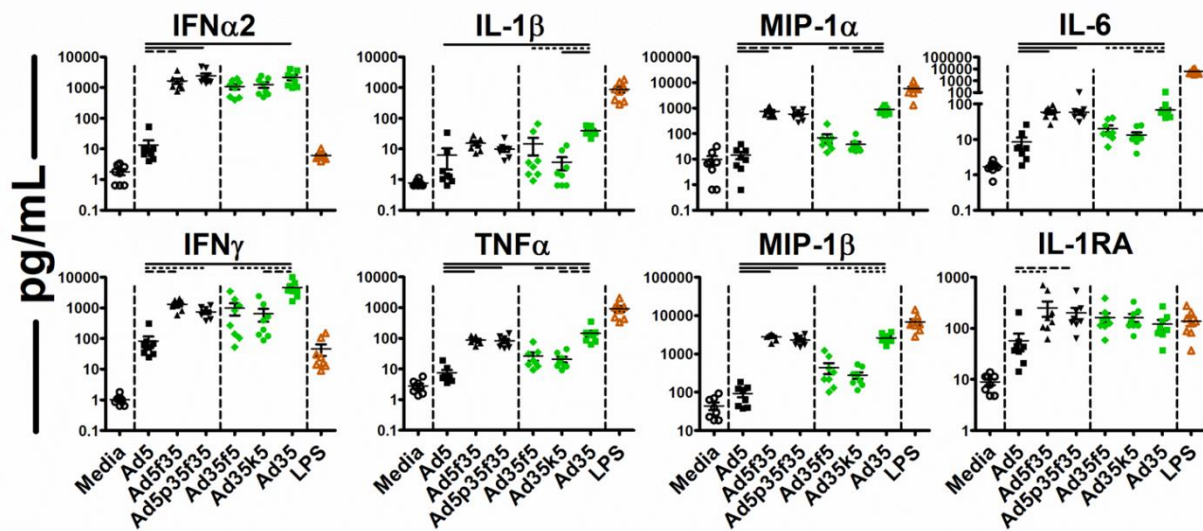


Figure 2.4. Cytokine and chemokine responses elicited by chimeric Ad5/Ad35 vectors in human PBMC. Normal human PBMC (n=8/group) were stimulated for 24h in the presence of chimeric Ad5/Ad35 vectors, and cytokine and chemokine levels were measured by luminex assays. LPS (1 ng/mL) was included as the positive control. Data are represented as individual measurements with mean responses \pm SEM. Bars indicate p<0.05 (dotted line), p<0.01 (dashed line), and p<0.001 (solid line) using Kruskal-Wallis tests with Dunn's correction for multiple comparisons.

Fiber-receptor interactions are required for Ad35 and Ad26 stimulation of human PBMC. We next assessed whether fiber interactions with the CD46 receptor triggered the robust innate immune stimulation by Ad35 and Ad26 vectors. Human PBMC (n=4-8/group) were pre-incubated with 10 µg/mL of the anti-CAR monoclonal antibody RmcB (40) or the anti-CD46 monoclonal antibodies 13/42 and M177 (41, 42) and were then stimulated with Ad35 and Ad26 as described above. As expected, IFN-α2 and IFN-γ levels induced by Ad35 and Ad26 vectors were not affected by RmcB. In contrast, Ad35 induced lower levels of IFN-α2 and IFN-γ in the presence of 13/42 ($p<0.05$), and Ad26 elicited dramatically lower levels of IFN-α2 and IFN-γ following preincubation of cells with either 13/42 ($p<0.05$) or M177 ($p<0.05$) (Figure 2.5). No cytokine responses were observed following incubation of cells with the mAbs alone (data not shown). These results support previous reports from our laboratory and others showing that CD46 is a primary cellular receptor for Ad35 and Ad26 (1, 12). These data demonstrate that fiber-receptor binding contributes substantially to the innate immune stimulation by these vectors. The greater effect of CD46 blockade on innate responses triggered by Ad26 as compared with Ad35 may reflect subtle differences in primary receptor interactions and/or secondary receptor usage by these vectors.

Multiple cellular subsets are stimulated by Ad35 and Ad26. To explore the contributions of various cellular subsets to innate cytokine secretion following Ad35 and Ad26 stimulation, we depleted specific cell subsets from healthy human PBMC (n=6/group) and repeated the cytokine assays as shown in Figure 3. T cells, B cells, NK cells, monocytes/macrophages (MonoMac), myeloid dendritic cells (mDC), or plasmacytoid dendritic cells (pDC) were depleted by magnetic bead separation, and effective depletion was confirmed by flow cytometry (data not shown). PBMC depleted of these various cell subsets were then incubated with Ad35 or Ad26 vectors, and cytokine and chemokine responses were assessed.

Depletion of T cells resulted in complete abrogation of IFN- γ induction and partial reduction of IL-1RA, whereas depletion of pDC led to substantial reduction of IFN- α 2, TNF- α and IL-1RA (Figure 2.6).

Depletion of monocytes and macrophages resulted in a pronounced reduction of proinflammatory markers IL-1RA, MIP-1 α and MIP-1 β (Figure 2.6). These data suggest that multiple cellular subsets contribute to the overall milieu of innate cytokines triggered by Ad vectors.

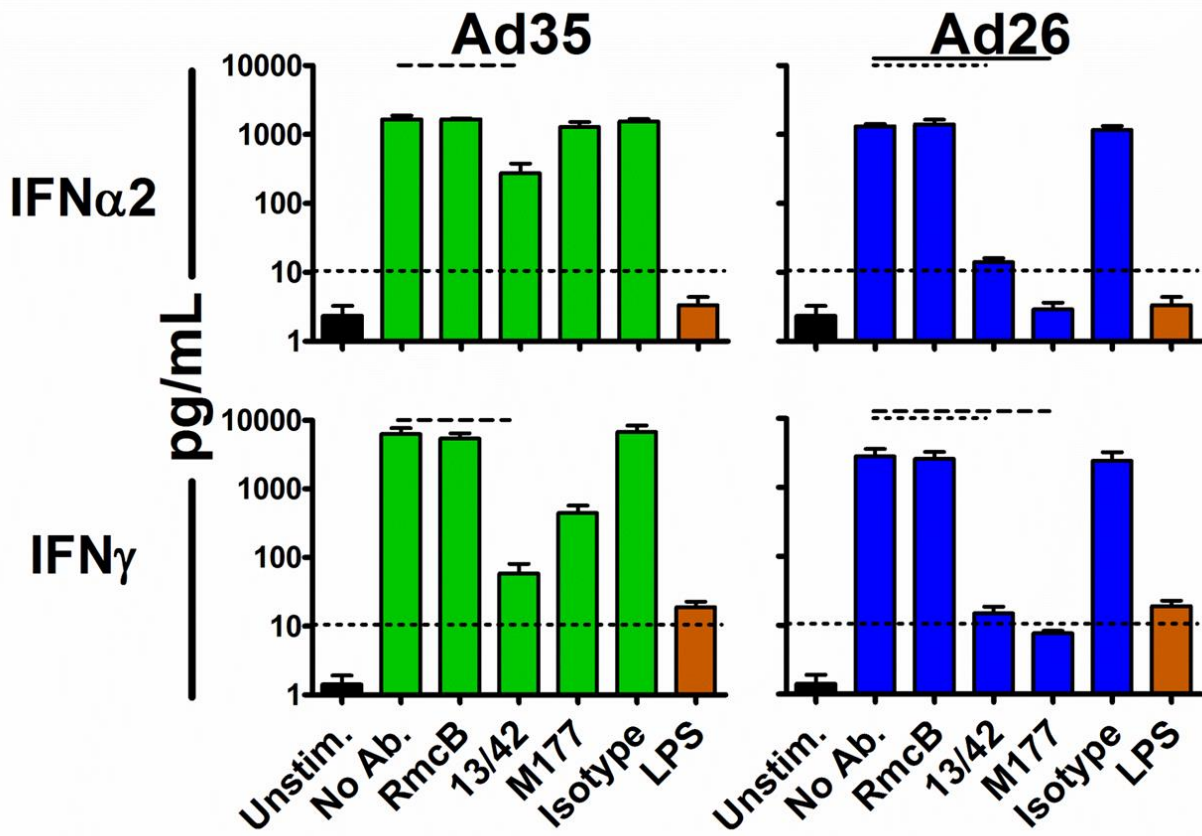


Figure 2.5. Interferon induction by Ad35 and Ad26 in the presence of anti-CAR or anti-CD46 blocking mAbs. Normal human PBMC ($n=4-6/\text{group}$) were preincubated with $10\text{ }\mu\text{g/mL}$ anti-CAR (RmcB) or anti-CD46 (13/42, M177) for 1 h and then stimulated with 10^3 vp/cell Ad35 or Ad26 vectors. IFN- α 2 and IFN- γ levels were measured 24h following stimulation by luminex assays. LPS (1 ng/mL) was included the positive control. Data are represented as mean responses \pm SEM. Bars indicate $p<0.05$ (dotted line), $p<0.01$ (dashed line), and $p<0.001$ (solid line) using Kruskal-Wallis tests with Dunn's correction for multiple comparisons.

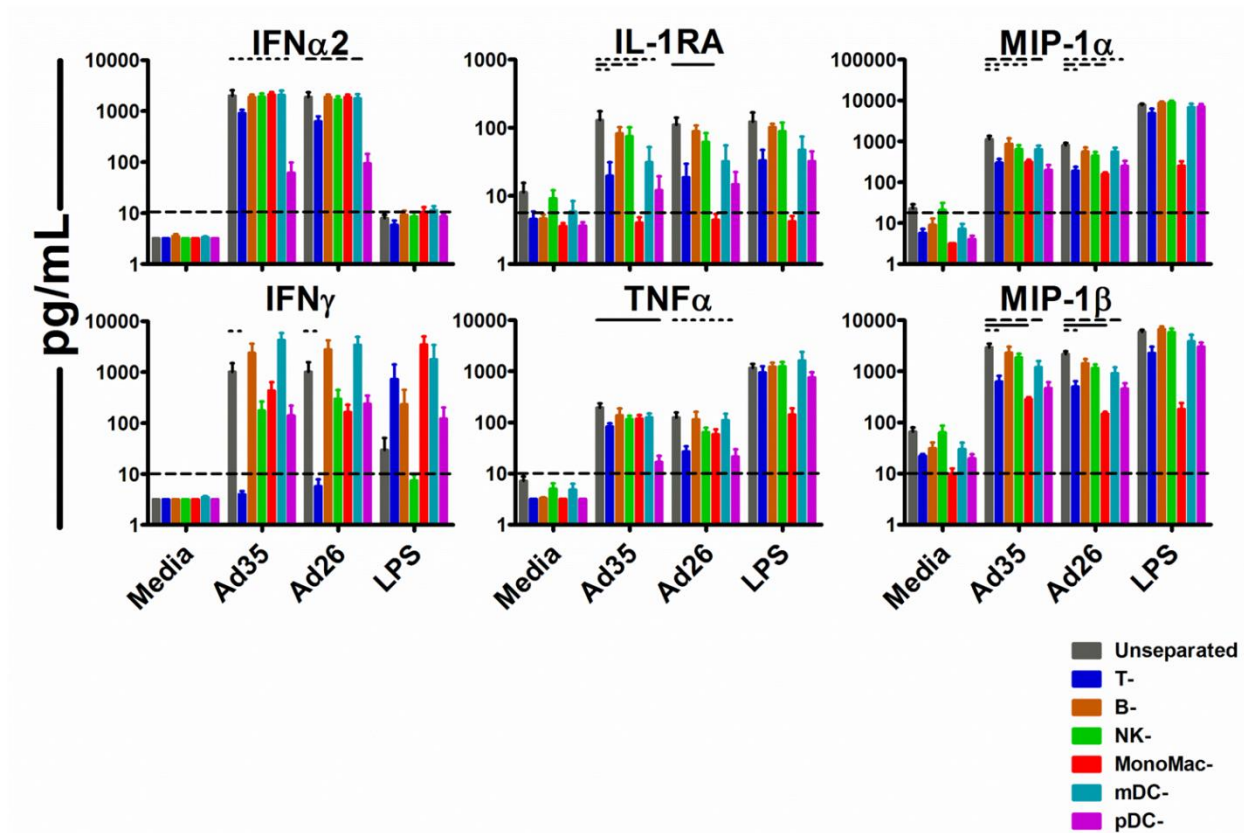


Figure 2.6. Cytokine and chemokine responses elicited by Ad vectors in normal human PBMC depleted of various cell populations. Normal human PBMC ($n=6/\text{group}$) were depleted of the indicated cell subsets by magnetic bead separation, and depletion was confirmed by flow cytometry (data not shown). PBMCs were then stimulated with 10^3 vp/cell of Ad35 or Ad26 vectors and cytokine and chemokine responses were measured after 24 h stimulation by luminex assays. LPS (1 ng/mL) was included as positive control. Data are represented as means \pm SEM, and unseparated versus depleted cell populations were compared. Bars indicate $p<0.05$ (dotted line), $p<0.01$ (dashed line), and $p<0.001$ (solid line) Kruskal-Wallis tests with Dunn's correction for multiple comparisons.

DISCUSSION

The innate immune profiles induced by various serotype Ad vaccine vectors remain poorly understood and have not previously been characterized in nonhuman primates. Here we demonstrate that the alternative serotype vectors Ad35, Ad26, and Ad48 induce substantially greater innate cytokine responses than Ad5 following vaccination of rhesus monkeys. In particular, Ad35, Ad26, and Ad48 induce higher levels of anti-viral and proinflammatory cytokines than Ad5, characterized by higher levels of IFN- γ , IP-10, I-TAC, IL-1RA, and IL-6.

Previous reports have characterized innate cytokine profiles of Ad vectors in mice (23, 30, 43) and *in vitro* (20, 21, 24, 25, 34, 44, 45). For example, several studies have reported that Ad35 induces higher levels of the co-stimulatory markers CD80 and CD40 and interferons than Ad5 *in vitro* in human DCs (25), as well as higher levels of IL-10 and reduced proliferation in T cells co-cultured with DCs (20). Studies in mice have suggested increased proinflammatory cytokine induction by Ad5 relative to CD46-utilizing vectors (22) which differs substantially from our findings in rhesus monkeys. Moreover, a recent study demonstrated that Ad35 and Ad28 induce high levels of IFN- α following immunization of mice (21). In contrast, we observed only minimal IFN- α secretion by Ad35 and Ad26 in rhesus monkeys. Taken together, these results demonstrate the importance of studying innate immune profiles of Ad vectors in primates rather than mice, which lack the cellular receptor CD46 distribution found in primates (37). This is particularly important for evaluating the innate cytokine responses elicited by Ad35, Ad26, and Ad48 that appear to be triggered largely by fiber-receptor binding (Figures. 2.4 & 2.5).

Fiber-receptor interactions are critical but do not fully explain the observed differences in innate immune triggering. In addition to its role as a receptor to certain species B and D adenoviruses, CD46 has a role in the binding complement components C3b and C4b (46, 47), and due to differential splicing may be immunosuppressive or immunostimulatory upon ligand binding (48, 49). Additionally, the

degree of CD46 binding may influence ligand endocytic trafficking fate, as well as the induction of autophagy (50, 51). As such, Ad35, Ad26, and Ad48 binding to CD46 may target them to endosomal compartments rich in pattern recognition receptors that could then influence innate immune responses differently from those induced by Ad5. The mechanism by which CD46 binding influences Ad vector innate stimulation thus warrants further exploration.

Our results also suggest that Ad capsid components such as hexon also contribute to innate stimulation. In particular, we observed a distinct day 7 peak of cytokine secretion in monkeys that received Ad48 and Ad5HVR48, which only share common hexon HVRs. The ability of the hexon HVRs to influence innate immune stimulation may reflect their role in binding serum factors (27), endocytic localization of virus (52), or other possible mechanisms.

In summary, our data demonstrate for the first time that the innate cytokine responses triggered by Ad35, Ad26, and Ad48 are substantially greater than those induced by Ad5 in rhesus monkeys. Specifically, the alternative serotype vectors that utilize CD46 as a primary cellular receptor induce more potent anti-viral and proinflammatory cytokine responses following vaccination than Ad5 that utilizes CAR. The in vivo characterization of these innate cytokine profiles contributes substantially to our understanding of these Ad vectors, which are currently being evaluated as candidate vaccine vectors in human clinical trials. Further studies assessing the impact of these profoundly different innate immunologic milieus on the subsequent generation of adaptive immune responses are therefore warranted.

MATERIALS AND METHODS

Viruses: E1/E3 deleted vectors Ad5, Ad35, Ad26 and Ad48 vectors expressing SIV antigens no transgene were produced as previously described (1, 39).

Cells: Normal human blood was collected in the presence of sodium heparin and processed by Ficoll-hypaque gradient method as previously described (53). Cells were resuspended in R10 medium (RPMI, 10%FCS, 50 u/mL Penicillin, 50 µg/mL Streptomycin) at a concentration of 1×10^6 cells/mL and utilized in *in vitro* assays. All studies involving human subjects were approved by the Beth Israel Deaconess Medical Center Institutional Review Board (IRB).

Antibodies: Anti-CAR mAb RmcB (Millipore, Billerica, MA) and anti-CD46 mAbs 13/42 (LifeSpan Biosciences, Seattle, WA) and M177 (Hycult Biotechnology, Plymouth Meeting, PA), as well as anti-KLH Mouse IgG isotype control (R&D Systems, Minneapolis, MN) were washed 3x with 1 mL unsupplemented DPBS and concentrated to 1 µg/µL by centrifugation at 3,000 rpm in Amicon Ultra-4 Centrifugal Filters (30kD MW) (Millipore, Billerica, MA) and stored at 4°C for immediate use. Flow cytometry antibody panels included CD3-allophycocyanin (UCHT1), CD16-fluorescein isothiocyanate (3G8), CD123-peridinin chlorophyll protein-Cy5.5 (9F5), CD11c-phycoerythrin (B-ly6), CD56-phycoerythrin-Cy7 (B159), CD19-V450 (HIB19), CD14- allophycocyanin-Cy7 (MΦP9) (BD Biosciences, San Diego, CA) and HLA-DR-AlexaFluor700 (LN3) (eBioscience, San Diego, CA).

In Vitro Cytokine Stimulation Assay: 1×10^6 PBMC were incubated with 1,000 vp/cell of the indicated adenovirus vector. In certain experiments, cells were pre-incubated with mAbs for 1 hr prior to infection. Cells were cultured at 37°C and supernatants were harvested 24 h following infection by centrifugation at 1,400 rpm for 5 minutes. Supernatants were analyzed utilizing Millipore Milliplex Map Human Cytokine/Chemokine magnetic luminex (Millipore, Billerica, MA) and Life Technologies Cytokine Human 30-Plex Panel (Life Technologies, Grand Island, NY) as per the manufacturer's protocols. Luminex

data were analyzed on BioPlex 200 instrument running Bioplex Manager 4.1 (Bio-Rad, Hercules, CA) with 80%-120% standard acceptance range. Data were graphed using Graph Pad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA). Means between groups were compared using Kruskal-Wallis tests with Dunn's correction for multiple comparisons and plotted as mean \pm SEM.

Magnetic Cell Separation: PBMC were depleted of CD3, CD14, CD19, CD56, CD1c, or BDCA-1 positive cell fractions by use of Magnetic MicroBeads according to manufacturer's protocol (Miltenyi Biotec, Auburn, CA). Depleted populations were confirmed by flow cytometry on LSRII and BD FACSDiva v.6.1.1 (BD Biosciences). Results were analyzed using FlowJo v.8.8.6 (Tree Star, Inc., Ashland, OR).

Non-Human Primate Ad Vector Vaccination: Rhesus monkeys (n=4-8/group) were immunized with 3×10^{10} vp Ad5, Ad35, Ad26, Ad48, or Ad5HVR48 expressing SIV Gag/Pol/Env antigens. Serum was collected on days 0, 1, 3, 7, 10, 14 and 28 following vaccination, thawed on ice, and inactivated with 0.05% Tween-20 for 15min at room temperature. Samples were then run on Milliplex Non-Human Primate 23plex Premix (Millipore) or Invitrogen Monkey Cytokine 28plex (Life Technologies), VeriKine Cynomolgus/Rhesus Interferon-Alpha Serum ELISA (PBL InterferonSource, Piscataway, NJ), and Human CXCL10/IP-10 Quantikine ELISA (R&D Systems, Minneapolis, MN) according to manufacturer's protocols, analyzed and graphed as described above. All studies involving rhesus monkeys were approved by the Harvard Medical School Institutional Animal Care and Use Committee (IACUC).

Data Analysis and Statistical Methods: Concentrations of cytokines and chemokines were obtained from luminex assays using a 5 parameter logistic model. Results of *in vitro* PBMC studies were compared by Kruskal-Wallis tests with Dunn's correction for multiple comparisons. Total cytokine induction *in vitro* was assessed by group average fold induction versus media controls. Cytokine induction *in vivo* was assessed as fold change of cytokine over average baseline level of all monkeys. Group fold change over averaged baseline was compared to monkeys vaccinated with Ad5 by Mann-Whitney U tests.

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**Chapter 3: Vaccination with an Adenovirus 26 Vector in Humans Induces a Robust but Transient
Systemic Antiviral and Proinflammatory Cytokine and Chemokine Response**

This chapter is based on samples and studies done as part of the Integrated Preclinical/Clinical AIDS Vaccine Development Program (IPCAVD) 003 study.

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INTRODUCTION

Adenoviral vectors have found wide utility in vaccinology as a platform for vaccination against many infections and cancers (1, 2). In particular, Adenovirus (Ad) 5 as well as “alternative serotype” Ad’s such as Ad35 and Ad26 vectors have been utilized in pre-clinical and clinical studies with the goal of generating vectors capable of eliciting protective immunity against HIV (3). Several studies in murine and non-human primate pre-clinical models have indicated that Ad vectors can differ substantially in their ability to elicit cellular and humoral immune responses (4–6). In particular, Ad35 and Ad26 have been shown to elicit more polyfunctional CD8⁺ T-cell responses relative to Ad5 vectors. Furthermore, these differences in adaptive immune phenotype formation are likely important for determining the construct efficacy at inducing protective immune responses against HIV, as suggested by the finding that heterologous vaccination with an Ad5 prime and either Ad35 or Ad26 boost reduced peak and setpoint viral loads in an SIVmac329 infection model in Rhesus monkeys as compared to a homologous Ad5/Ad5 regimen. Understanding the means by which these differential adaptive immune phenotypes are generated and their influence on resultant protection from pathogenic challenge will be instrumental in further vaccine design optimization.

Ad vectors can differ substantially in their basic biological properties such as primary receptor usage, dendritic cell transduction, and usage of Pattern Recognition Receptor (PRR) stimulation (7–9). Previous studies from our lab and others have indicated that Ad vectors can differ not only in their ability to elicit adaptive immune phenotypes following vaccination, but also substantially differ in their innate immune stimulatory profiles (6, 10). Results obtained both murine and non-human primate model systems indicated that the CD46-utilizing vectors Ad35, Ad26, and Ad28 induce a markedly higher level of systemic antiviral and proinflammatory cytokines and chemokines relative to Ad5 following vaccination. This difference in innate immune stimulation may influence elicited adaptive immune

phenotypes and protection, and as such are an important component in understanding the relative immunogenicity of these vectors. However, despite the relative importance of innate immune stimulation in determining adaptive immune phenotypes, the innate stimulatory properties of these vectors, in particular Ad35 and Ad26, remain to be well characterized in a human vaccination setting. Characterization of the innate stimulatory properties of a CD46-utilizing Ad vector in human volunteers would confirm pre-clinical observations of Ad vector elicited innate immune stimulation phenotypes and extend their utility to understanding vaccine-elicited adaptive immune responses.

In this study, we characterized the innate immune stimulatory properties of an Ad26.HIVEnvA vector in 18 Human volunteers. Systemic cytokine and chemokine profiles were utilized as a surrogate marker for innate immune activation. Vaccination of both Ad26 seronegative and seropositive individuals with an Ad26.HIVEnvA vector resulted in a transient but robust induction of several antiviral and proinflammatory cytokines and chemokines 1 day following vaccination, which largely subsided by day 3 following vaccination. Volunteer Ad26 serostatus at the time of vaccination was not found to alter the identity of cytokines induced, but Ad26 seropositive individuals displayed a trend toward reduced induction of elicited cytokines and chemokines at peak levels. These studies indicate that Ad26 induces a systemic antiviral and proinflammatory cytokine milieu immediately following vaccination, in agreement with our previous findings in the rhesus monkey pre-clinical model. Further evaluation as to the influence of this cytokine milieu on elicited adaptive immune phenotypes may yield important insights into vaccine elicited adaptive immune phenotypes and protection from subsequent pathogenic challenge.

RESULTS

We initiated studies by assessing systemic cytokine and chemokine responses in 24 Human volunteers. Volunteers were separated into 3 groups. The first group consisted of 12 individuals who were Ad26 seronegative at the time of vaccination and who received 5×10^{10} vp Ad26 expressing HIV.EnvA intramuscularly (i.m.). The second group consisted of 6 individuals who were Ad26 seropositive at the time of vaccination and who similarly received 5×10^{10} vp Ad26 expressing HIV.EnvA i.m. A third group was composed of 6 individuals who received a sham vaccination. Sera were collected on days 0, 1, 3, 7, and 28 following vaccination and then systemic levels of 72 cytokines and chemokines were assessed utilizing Luminex protocols as previously described (10). Analysis of systemic cytokine and chemokine responses in both groups of volunteers who received the Ad26.HIVEnvA immunization revealed a robust but transient increase in systemic levels of many antiviral and proinflammatory cytokines and chemokines at 1 day following vaccination (Figure 3.1A). This transient increase in systemic cytokine and chemokine levels largely subsided by day 3, and completely by day 7, following vaccination (Figure 3.1A). This increase in systemic cytokine and chemokine levels was most pronounced in those donors who were Ad26 seronegative at the time of vaccination. Relative to aggregate baseline levels for all donors, volunteers who were Ad26 seronegative at the time of vaccination with Ad26.HIVEnvA displayed a pronounced induction of several antiviral and proinflammatory cytokines and chemokines such as interferon gamma (IFN- γ) (2.1x fold greater levels relative to aggregate day 0 baseline; $p=0.0148$, Kruskal-Wallis Tests), interferon gamma Induced Protein 10 (IP-10) (11.7x fold greater; $p<0.0001$), interferon-inducible T-cell alpha chemoattractant (I-TAC) (9.9x fold greater; $p<0.0001$), interleukin-1 receptor antagonist (IL-1RA) (2.9x fold greater; $p=0.0023$), tumor necrosis factor alpha (TNF α) (1.5x fold greater; $p=0.0034$), and monocyte chemotactic protein-1 (MCP-1) (2.2x fold greater; $p=0.0086$) (Figure 3.1A). Similar trends were observed with donors who were Ad26

seropositive at the time of Ad26.HIVEnvA vaccination for antiviral cytokines and chemokines, though many of these trends failed to reach statistical significance. Vaccination of Ad26 seropositive volunteers with Ad26.HIVEnvA resulted in systemic increases relative to baseline aggregate levels of IFN- γ (1.6x fold greater induction relative to aggregate day 0 baseline; $p=0.1024$, Kruskal-Wallis Tests), IP-10 (5.2x fold greater induction; $p=0.0004$), I-TAC (1.3x fold greater induction; $p=0.2040$) (Figure 3.1A). Analysis of median fold change over aggregate baseline levels for all cytokines analyzed for each group showed a broad and robust induction of many antiviral and proinflammatory cytokines and chemokines for both Ad26 seronegative and Ad26 seropositive vaccinees at 1 day following immunization (Figure 3.1B). Both groups of volunteers who received an Ad26 immunization displayed induction of IFN- γ , interleukin-10 (IL-10), IL-1RA, Interleukin 1 alpha (IL-1 α), MCP-1, TNF α , monocyte chemoattractant protein 2 (MCP-2), TNF-related apoptosis-inducing ligand (TRAIL), monokine induced by gamma interferon (MIG), I-TAC, and macrophage inflammatory protein-3-beta (MIP-3 β) (Figure 3.1B). Overall, Ad26 serostatus had no influence on the identity of cytokines and chemokines elicited, with donors possessing Ad26 seropositivity at the time of vaccination displaying reduced median fold induction for a subset of cytokines and chemokines, including IFN- γ , TRAIL, and I-TAC (Figure 3.1B). These data indicate that intramuscular vaccination with an Ad26 vector leads to a pronounced but transient induction of many antiviral and proinflammatory cytokines and chemokines, in agreement with previously reported results following intramuscular vaccination of Rhesus monkeys with an Ad26 construct.

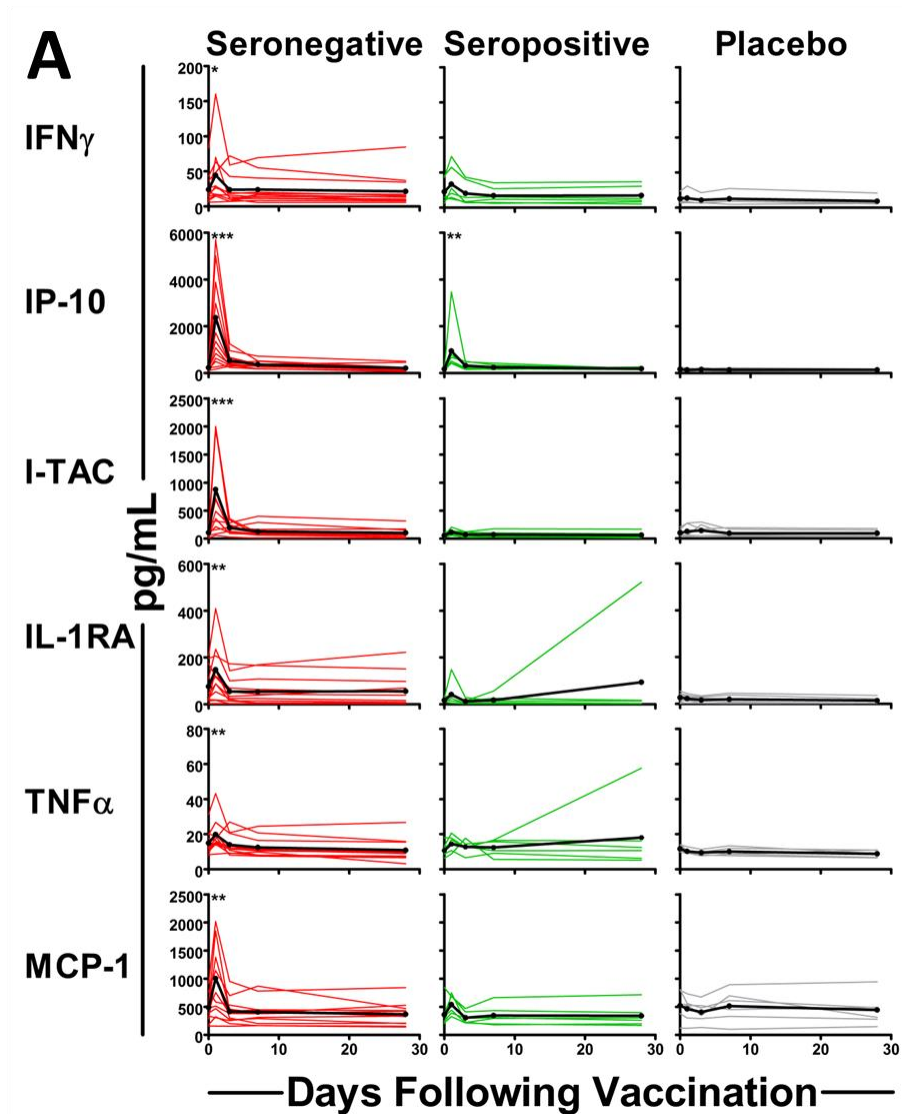


Figure 3.1. Serum concentrations of cytokines and chemokines in human volunteers following Ad26 vaccination. Human volunteers (n=6-12/group) were vaccinated i.m. with 3×10^{10} Ad26 vector encoding HIV.EnvA antigen. An additional group (n=6) was placebo vaccinated. Sera were collected on days 0, 1, 3, 7 and 28 following vaccination, and systemic levels of cytokines and chemokines were analyzed by Luminex assays. (A) Systemic levels of selected cytokines and chemokines following vaccination with Ad26 or placebo, with individual levels shown as colored lines and group median over time shown with black lines. Stars indicate cytokine peak detected (d1 vs. d0 Wilcoxon Rank-Sum Tests; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

B

	Seronegative	Seropositive	Placebo		Seronegative	Seropositive	Placebo	Fold Induction	
EGF				TNFA				0.75-1.25	
FGF-2				TNFB				1.25-1.5	
Eotaxin				VEGF				1.5-3	
TGF- α				Eotaxin-2				3-10	
G-CSF				MCP-2				>10	
Flt-3L				BCA-1					
GM-CSF				MCP-4					
Fractalkine				I-309					
IFN α 2				IL-16					
IFN γ				TARC					
GRO				6CKine					
IL-10				Eotaxin-3					
MCP-3				LIF					
IL-12p40				TPO					
MDC				SCF					
IL-12p70				TSLP					
IL-13				IL-33					
IL-15				IL-20					
IL-17				IL-21					
IL-1RA				IL-23					
sIL-2RA				TRAIL					
IL-1 α				CTACK					
IL-9				SDF-1 α + β					
IL-1b				ENA-78					
IL-2				MIP-1d					
IL-3				IL-28A					
IL-4				M-CSF					
IL-5				MIG					
IL-6				GCP-2					
IL-7				I-TAC					
IL-8				MIP-3b					
IP-10				MIP-3a					
MCP-1				Lymphotactin					
MIP-1a				IL-11					
MIP-1b				IL-29					

Figure 3.1., continued. (B) Median fold induction for all cytokines and chemokines at day 1 post vaccination with Ad26 or placebo vaccination in rhesus human volunteers. Values shown are group mean fold induction over the averaged aggregate baseline.

We next sought to analyze the influence of Ad26 serostatus at the time of vaccination on the levels of elicited cytokines and chemokines following immunization. Fold induction over averaged aggregate baseline levels at day 0 was calculated for each donor and cytokine and elicited levels compared between groups. Similar to our findings above, vaccination of Ad26 seronegative volunteers induced a robust increase in systemic levels relative to the sham vaccinated group for several antiviral and proinflammatory cytokines and chemokines such as IFN- γ (2.1x vs. 0.7x fold induction relative to day 0 aggregate baseline; $p=.011$, Kruskal-Wallis Tests), I-TAC (9.4x vs. 1.4x; $p=0.0056$), IL-1RA (3.0x vs. 0.5x; $p=0.0017$), IL-1 α (3.1x vs. 0.5x; $p=0.003$), TNF- α (1.5x vs. 0.8x; $p=0.0002$) and MIP-3 β (1.9x vs. 1.0x; $p=0.0002$) (Figure 3.2). Significantly greater fold induction was observed for both Ad26 seronegative and Ad26 seropositive vaccinees relative to sham vaccines for a subset of cytokines and chemokines including IP-10 (11.7x & 5.2x vs. 0.7x, respectively; $p=0.0002$ & $p=0.0022$, respectively), MCP-2 (3.0x & 2.5x vs. 1.0x, respectively; $p=0.003$ & $p=0.041$, respectively), TRAIL (3.7x & 3.6x vs. 0.9x, respectively; $p=0.0047$ & $p=0.0043$, respectively), and MIG (2.3x & 1.1x vs. 0.6x, respectively; $p=0.0047$ & $p=0.0087$, respectively) (Figure 3.2). As observed above, lower elicitation of cytokines and chemokines was observed in the Ad26 seropositive vaccinees relative to Ad26 seropositive vaccinees for IL-1RA (0.9x vs. 3.0x fold induction over day 0 aggregate baseline, respectively; $p=0.031$) and I-TAC (1.3x vs. 9.4x, respectively; $p=0.011$), with a similar trend being observed for several other cytokines and chemokines such as IL-1 α , IP-10, MCP-1, TNF- α , and MIG (Figure 3.2). These results indicate that Ad26 serostatus of vaccinees prior to Ad26 vaccination does not alter the identity of cytokines and chemokines elicited, but that serostatus may influence the magnitude of this induced response.

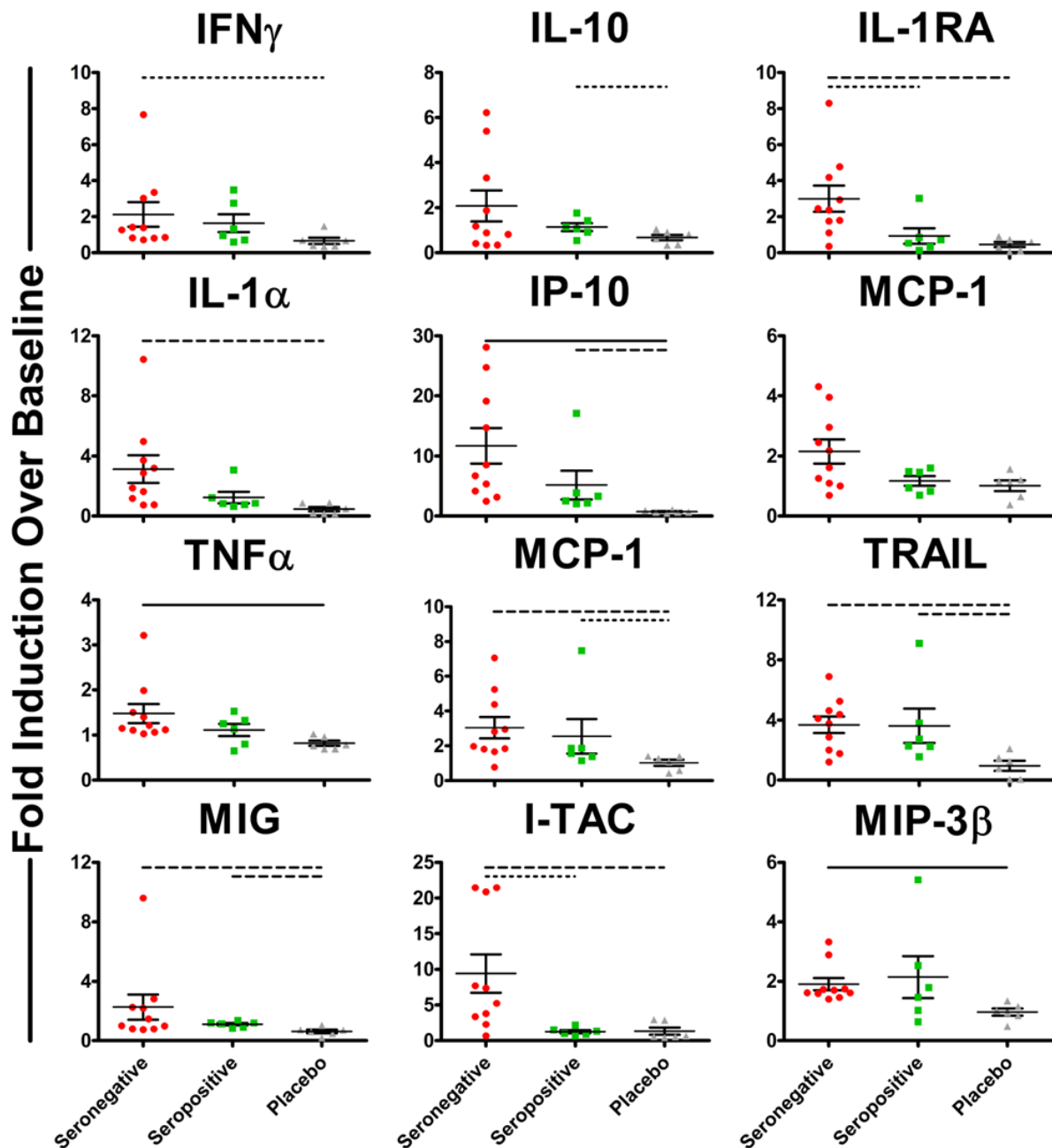


Figure 3.2. Peak cytokine and chemokine levels of Ad26 sero-positive/sero-negative volunteers following Ad26 vaccination. Peak cytokine and chemokine levels for selected analytes at day 1 following vaccination for Ad26- and placebo-vaccinated human volunteers. Data are shown as mean \pm SEM. Bars indicate P values of <0.05 (dotted lines), <0.01 (dashed line), or <0.001 (solid line) using Kruskal-Wallis tests.

DISCUSSION

Our results here show that the CD46-utilizing vector Ad26 was able to elicit a potent antiviral and proinflammatory cytokine and chemokine induction following intramuscular immunization in 18 human volunteers independent of an individual's Ad26 serostatus at the time of vaccination. This induction was transient, peaking at day 1 following vaccination and subsequently subsiding by day 3 post-vaccination. Furthermore, the identity of cytokines and chemokines elicited was unaltered by the presence of pre-existing humoral immune responses against Ad26. However, pre-existing neutralizing antibodies against Ad26 were able to reduce the overall fold induction over baseline for several cytokines and chemokines analyzed. Whether this reduction is proportional to the titer of pre-existing neutralizing Ad26 antibodies at the time of vaccination remains to be determined. Importantly, the observed peaks in cytokine and chemokine levels in vaccinated levels closely match both the kinetics and identity of cytokines and chemokines previously observed to be induced by Ad26 immunization in Rhesus monkeys (10). In both humans and Rhesus monkeys, intramuscular immunization with Ad26 induced a peak in systemic levels at day 1 post-vaccination of IFN- γ , IP-10, I-TAC, and IL-1RA. Additionally, similar to observations in the Rhesus monkey model, systemic increases of several cytokines such as IFN- α 2 and TNF- α were not detected. Together these data indicate that systemic cytokine and chemokine induction elicited by immunization with Ad26 in both humans and Rhesus monkeys are congruent. A complimenting analysis of innate cytokine and chemokine responses in Ad35 seronegative and seropositive human volunteers following Ad35 immunization would additionally enable correlation of innate immune stimulation capacity by Ad vectors with serostatus. The role of innate immune cytokine and chemokine responses in the formation of adaptive immune phenotypes elicited by Ad26 also warrants further investigation.

Innate immune cytokine signatures elicited by Ad26 following immunization likely play an influential role in the generation of vaccine-elicited adaptive immune responses. As mentioned above Ad26 has been previously shown in pre-clinical non-human primate as well as clinical human studies, to elicit high numbers of polyfunctional CD8⁺ T cells, a phenotype which has been correlated with lower viral loads in SIV model infection of rhesus monkeys (11, 12). Furthermore, it has been demonstrated that vaccination with Ad26 induces mucosal trafficking of effector memory T cells, potentially seeding the mucosal periphery with cells able to immediately extinguish an HIV infection (13). The systemic cytokine milieu described in this study is likely spill-over of a localized stimulatory event, as evidenced by a broader array of cytokines previously shown to be elicited in a human PBMC *in vitro* model system compared to *in vivo* levels. Various PBMC subsets primed in such an immunostimulatory local milieu may be imprinted with distinct adaptive phenotypes. For example, it has been previously demonstrated that CD8⁺ T cells primed in the presence of IFN- α display enhanced cytotoxic potential upon recognition of their cognate antigen (14). It is furthermore likely that the cytokine and chemokine responses presented here have several means by which they alter induced adaptive responses beyond these hypothetical mechanisms.

The results presented in these studies are important in light of prior studies suggesting immune activation following Ad5 vaccination to be influential in negatively shaping adaptive immune phenotypes, in particular conferring a statistically non-significant increase in HIV acquisition risk (15, 16). However, reports from several groups have indicated that pre-existing immunity to an Ad5 vector does not lead to increased CD4⁺ T cell target populations in the distal mucosa and likely does not account for any observed enhancement phenotype (17–22). As indicated by studies from our group as well as others, significant differences exist between Ad vectors in their immunological properties (4, 6, 10, 23). Therefore, the innate immune stimulatory signature observed in these studies may potentially influence

adaptive immune response formation differently than innate immune influences on adaptive immunity suggested by prior studies utilizing Ad5, and these differences on resultant vaccine-elicited immune responses in humans warrants further investigation.

These studies indicate that Ad26 induces a potent antiviral and proinflammatory cytokine and chemokine milieu following vaccination of human volunteers, regardless of volunteer Ad26 serostatus at the time of vaccination. Importantly, these observations closely mirror previous observations from our group showing similar cytokine and chemokine responses following Ad26 vaccination of rhesus monkeys and *in vitro* stimulation of human PBMC (10). Therefore, pre-clinical rhesus monkey model vaccination studies offer a congruent system in which to probe the links between innate immune activation elicited immediately following vaccination and subsequent development of adaptive immune responses. Greater understanding of the mechanisms and consequences of these links between vector elicited innate immune responses and vaccine-elicited adaptive immune phenotypes will be important for further vector optimization as well as understanding the means by which possible correlates of vaccine-induced protection are elicited.

MATERIALS AND METHODS

Viruses: E1/E3 deleted Ad26 was produced as previously described (4).

Human Ad Vector Vaccination: Human volunteers (6-12/group) were immunized with 5×10^{10} vp Ad26 expressing HIV.EnvA. Serum was collected on days 0, 1, 3, 7 and 28 following vaccination, thawed on ice, and inactivated with 0.05% Tween-20 for 15min at room temperature. Samples were then analyzed with Milliplex Human Cytokine/Chemokine panels I, II, and III (Millipore, Billerica, MA, USA) according to manufacturer's protocols. Luminex data were analyzed on BioPlex 200 instrument running Bioplex Manager 4.1 (Bio-Rad, Hercules, CA) with 80%-120% standard acceptance range. Data were graphed using Graph Pad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA). All studies involving human volunteers were approved by the Beth Israel Deaconess Medical Center Institutional Review Board (IRB)

Data Analysis and Statistical Methods: Concentrations of cytokines and chemokines were obtained from luminex assays using a 5 parameter logistic model. Day one cytokine levels were compared to baseline levels on a per group basis and fold change induction over baseline comparisons between groups were analyzed using Kruskal-Wallis Tests.

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**Chapter 4: Late Endosomal Trafficking of Alternative Serotype Adenovirus Vaccine Vectors Augments
Antiviral Innate Immunity**

This chapter is based on the peer-reviewed publication:

Teigler, J.E., Kagan, J., and Barouch, D.H. 2013. Late Endosomal Trafficking of Alternative Serotype Adenovirus Vaccine Vectors Augments Antiviral Innate Immunity. Manuscript in submission at the time of writing.

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ABSTRACT

Adenovirus (Ad) vaccine vectors have found widespread use as vaccine platforms against multiple infections and cancers, and multiple serotypes have been shown to differ significantly in their biologic properties and immune phenotypes. Our laboratory and others have previously described differential innate immune stimulation elicited by various Ad serotypes. Here we show that Ad5 traffics rapidly to the nucleus following infection, whereas Ad35 and Ad26 accumulate in late endosomes between 2-8 hours post-infection. Innate immune cytokine elicitation by all Ad serotypes was abrogated by blockade of endosomal acidification, Cathepsin B and Caspase-1, suggesting that virus interactions with acid-dependent TLR sensors and inflammasome activation in late endosomes may trigger innate immunity. These data suggest a mechanism by which Ad vectors from various serotypes differentially trigger innate antiviral pathways via distinct intracellular trafficking to late endosomes.

INTRODUCTION

Given the limitations that have become evident with adenovirus serotype 5 (Ad5) vectors, alternative serotype Ad vectors have been developed from serotypes against which high-level humoral responses are less common in the global population, including Ad35 and Ad26 (1–3). Interestingly, in addition to circumventing high levels of Ad5 neutralizing antibodies, alternative serotype Ad vectors differ substantially from Ad5 in the phenotypes of innate and adaptive immune responses elicited by vaccination (1, 4, 5). Moreover, several alternative serotype Ad vector-based vaccines confer increased protection relative to Ad5 against pathogenic SIVmac251 challenges in rhesus monkeys (4, 6, 7).

Innate immune stimulation is a critical determinant for establishing the magnitude and phenotype of adaptive immune responses (8–11). Previously, our group and others have shown that Ad vectors differ markedly in their innate immune stimulatory properties (12, 13). In particular, Ad35 and Ad26 induce significantly higher levels of antiviral and proinflammatory cytokines and chemokines in serum from vaccinated rhesus monkeys and in fresh human PBMC as compared with Ad5 (12). However, the mechanism underlying these observed differences has not previously been determined.

Results from several groups have suggested that Toll-like receptor 9 (TLR9), late endosomal lysis, and/or differences in tropism may contribute to innate stimulation by Ad vectors (14–17). Previous reports utilizing a variety of cell systems have also shown that Ad vectors can utilize different endosomal trafficking pathways such as clathrin-mediated endocytosis or macropinocytosis, and they may exit either early or late endosomes (18–24). We therefore hypothesized that differential Ad vector trafficking and access to late endosomal pattern recognition receptors (PRR) might account for the differential innate stimulation elicited by these vectors.

In this study, we show that Ad35 and Ad26 accumulate in the late endosomal compartment more extensively than Ad5 at 2–8 hours following infection. Innate immune stimulation by all Ad vectors

was sensitive to inhibitors of endosomal acidification, Cathepsin B, and Caspase-1. Thus, whereas Ad vectors stimulate common innate immune sensing pathways in the late endosome, marked differences in intracellular trafficking and access to this late endosomal compartment likely account for the different innate immune phenotypes elicited by these Ad vectors.

RESULTS

Ad35 and Ad26 accumulate to higher levels than Ad5 in the late endosomal compartment at 2-8 hours following infection. To assess the intracellular trafficking pathways for Ad5, Ad35, and Ad26, vectors were covalently labeled with the NHS-ester of Atto633 and tested for viability as previously described (20). Briefly, A549 cells were infected with an MOI of 5,000-50,000 viral particles (vp)/cell of fluorescently-labeled Ad vector, and colocalization was measured over time by confocal microscopy for the early endosome marker Early Endosome Antigen 1 (EEA1) or the late endosomal markers Lysosomal-Associated Membrane Protein 1 (LAMP1) or Mannose-6-Phosphate Receptor (M6P) by immunofluorescence histochemistry, and nuclei were labeled by DAPI (25–27). Viruses were measured for colocalization in a given endosomal/nuclear compartment by calculation of Manzel correlation coefficient, and differences between vector localization at a given time point were determined by Student's t tests.

Ad5 virions rapidly accumulated in the peri-nuclear region, with 72.6% of virions colocalizing with nuclear staining by 2 h post-infection (Figures 4.1A & 4.1B). Colocalization of Ad5 with the early endosomal marker EEA1 occurred only minimally and never exceeded 7% at any timepoint measured (Figure 4.1A). Ad5 virions also only minimally localized with late endosomes, as indicated by low levels of colocalization with LAMP1 or M6P at 2 h post infection (4.0% and 2.7% colocalization, respectively) (Figures 4.1A & 4.1B). These results indicate that Ad5 rapidly trafficked to the nucleus and only minimally accumulated in early and late endosomal compartments.

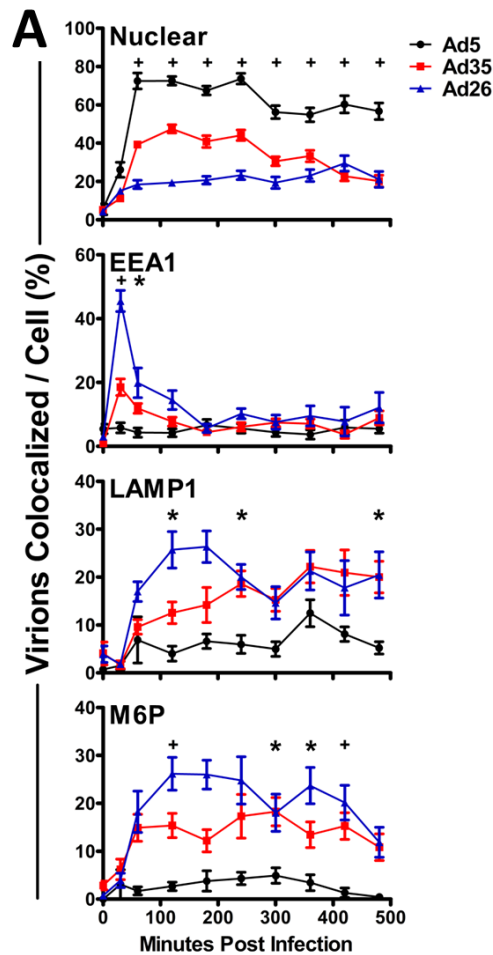


Figure 4.1. Ad35 and Ad26 accumulate more extensively than Ad5 in the late endosomal compartment. A549 cells were cold-synchronized for endocytosis and infected with 5,000-50,000 vp/cell of Atto633-labeled Ad5, Ad35, or Ad26. Cells were fixed in 4% Paraformaldehyde at various time points post infection and stained for Nucleus by DAPI and Early Endosomal Antigen 1 (EEA1), Lysosomal-associated membrane protein 1 (LAMP1), or Mannose-6-phosphate receptor (M6P) by immunofluorescence histochemistry. Cells were imaged at 630x magnification by confocal microscopy with z-stack slice depth of 0.5 μm and slice interval of 0.3 μm . A.) Percentage Ad5, Ad35, and Ad26 virions colocalized with Nuclei, EEA1, LAMP1, and M6P per cell following synchronized infection. Z-stack images (n=2-7 replicate experiments) analyzed for colocalization utilizing Volocity image analysis software. Compartments identified as $>3\times$ image standard deviation, and virions identified as particles $>0.078 \mu\text{m}^3$ with intensity $>3\times$ image standard deviation. Virions colocalization with compartments measured using Manzel correlation coefficients of >0.5 . Symbols indicate timepoints where both Ad35 and Ad26 colocalization values were both significantly different than Ad5, with the less significant value indicated (* $p<0.01$, $^+p<0.001$; Student's t tests).

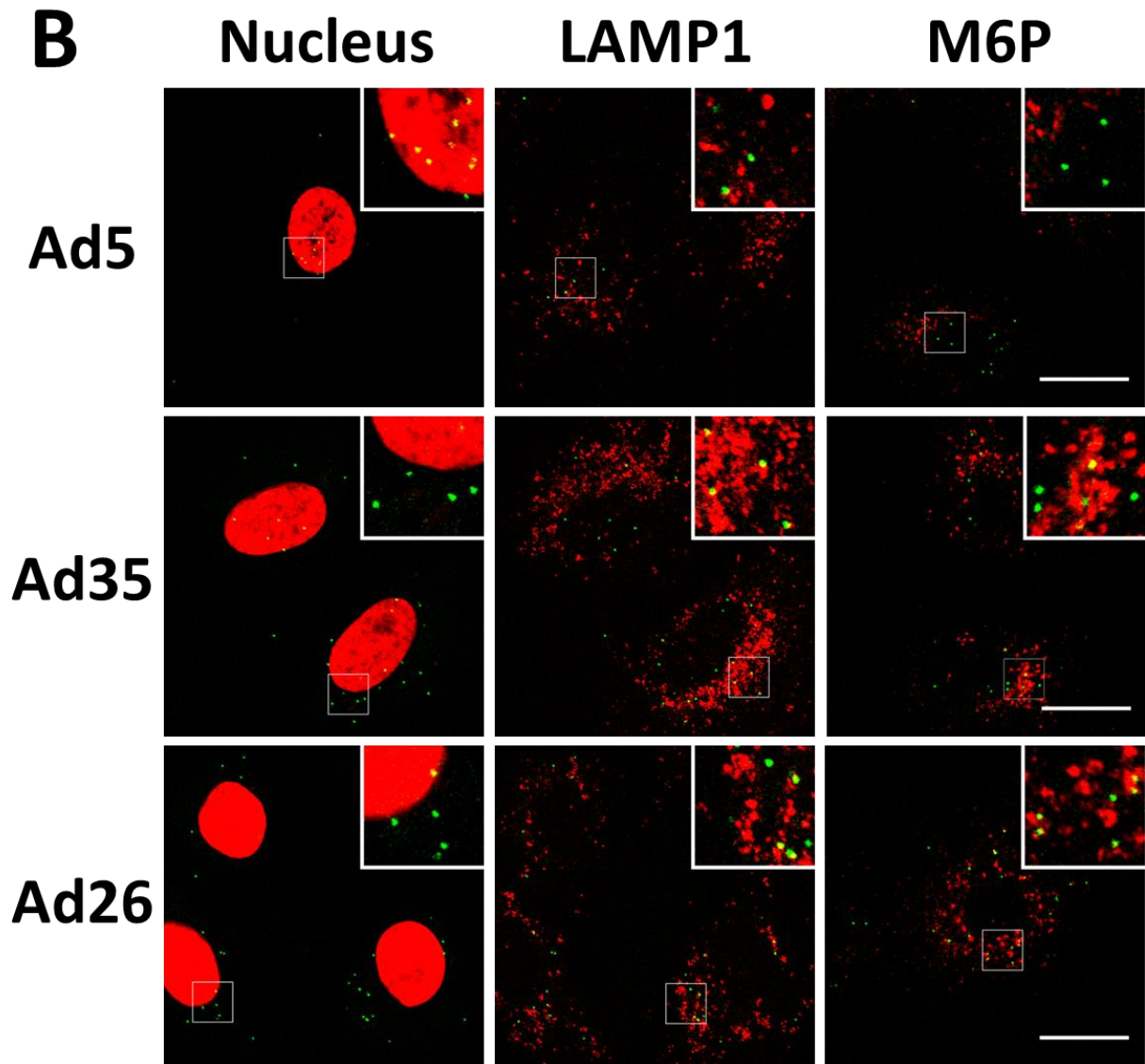


Figure 4.1., continued. B) Representative images of Ad5, Ad35, and Ad26 subcellular localization 2 h post-infection, with subpanel region indicated by gray outline. Indicated virus shown in green and indicated endosomal compartment or nucleus shown in red, with areas of overlap shown as yellow. Images processed using Fiji, scale bar = 20µm.

Ad35 and Ad26 exhibited marked differences in intracellular trafficking pathways as compared with Ad5. Ad35 and Ad26 displayed substantial but transient colocalization with the early endosomal marker EEA1 at 30 min (18.5% and 45.5% colocalization, respectively) and at 1 h (18.9% and 19.9% colocalization, respectively) post infection (Figure 4.1A). The colocalization of Ad35 and Ad26 with early endosomes was greater than that observed for Ad5 at both 30 min (3.2x and 7.8x fold greater colocalization, respectively; $p=0.0001$, $p<0.0001$, respectively; Student's t tests) and at 60 min (2.8x and 4.6x fold greater colocalization, respectively; $p=0.0082$, $p=0.0048$, respectively). At later time points, both Ad35 and Ad26 demonstrated substantial colocalization with the late endosomal marker LAMP1, with 12.6% and 25.7% of virions colocalizing with late endosomes at 2 h post-infection, respectively (Figures 4.1A & 4.1B). Ad35 and Ad26 also colocalized with the late endosomal marker M6P at 2 h post-infection (15.4% and 26.2% of virions, respectively) (Figures 4.1A & 4.1B). Both Ad35 and Ad26 accumulated in the late endosomal compartment to significantly higher levels than Ad5 at 2 h post infection as measured by both LAMP1 (3.1x and 6.4x fold greater colocalization, respectively; $p=0.0088$, $p=0.0001$, respectively) and M6P (5.8x and 9.8x fold greater colocalization, respectively; $p<0.0001$, both) colocalization. This association of Ad35 and Ad26 late endosomes persisted for all time points observed between 2-8 h post-infection. Taken together, these results indicate that Ad35 and Ad26 display a markedly different intracellular trafficking pattern as compared with Ad5, characterized by substantially greater association with late endosomes 2-8 hours post-infection.

Ad Innate immune stimulation is blocked by inhibitors of late endosomal acidification. Ad35 and Ad26 induce markedly higher levels of antiviral and proinflammatory cytokines and chemokines, including IFN- α , IFN- γ , IP-10, IL-6, and IL-1RA, than Ad5 in rhesus monkeys and human PBMC (12). Interestingly, these differences in innate immune cytokines and chemokines corresponded with the observed levels of Ad

vector trafficking to the late endosome. To probe the possible late endosomal innate sensing pathways the different Ad vectors may activate, we assessed the dependence of innate triggering by these vectors to late endosomal acidification. Fresh human PBMC (n=4/group) were isolated by ficoll-hypaque density gradient centrifugation and pre-incubated with the endosomal acidification inhibitors Bafilomycin A1 (BafA), Chloroquine, or Ammonium chloride for 1 h prior to infection with 1,000 vp/cell of Ad5, Ad35, or Ad26 (28, 29). Cytokine induction was measured 24 h following infection by Luminex assays as previously described (12).

Pre-incubation of human PBMC with BafA, Chloroquine, or Ammonium chloride markedly decreased induction of innate cytokines and chemokines by all the Ad vectors studied (Figure 2). This inhibition was most pronounced for IFN- α 2, with pre-incubation of PBMC with BafA, Chloroquine, or Ammonium chloride leading to reduced levels of IFN α 2 upon stimulation with Ad5, Ad35, or Ad26 (Figure 4.2). Together, these data indicate that innate stimulation by all three vectors was sensitive to inhibitors of late endosomal acidification.

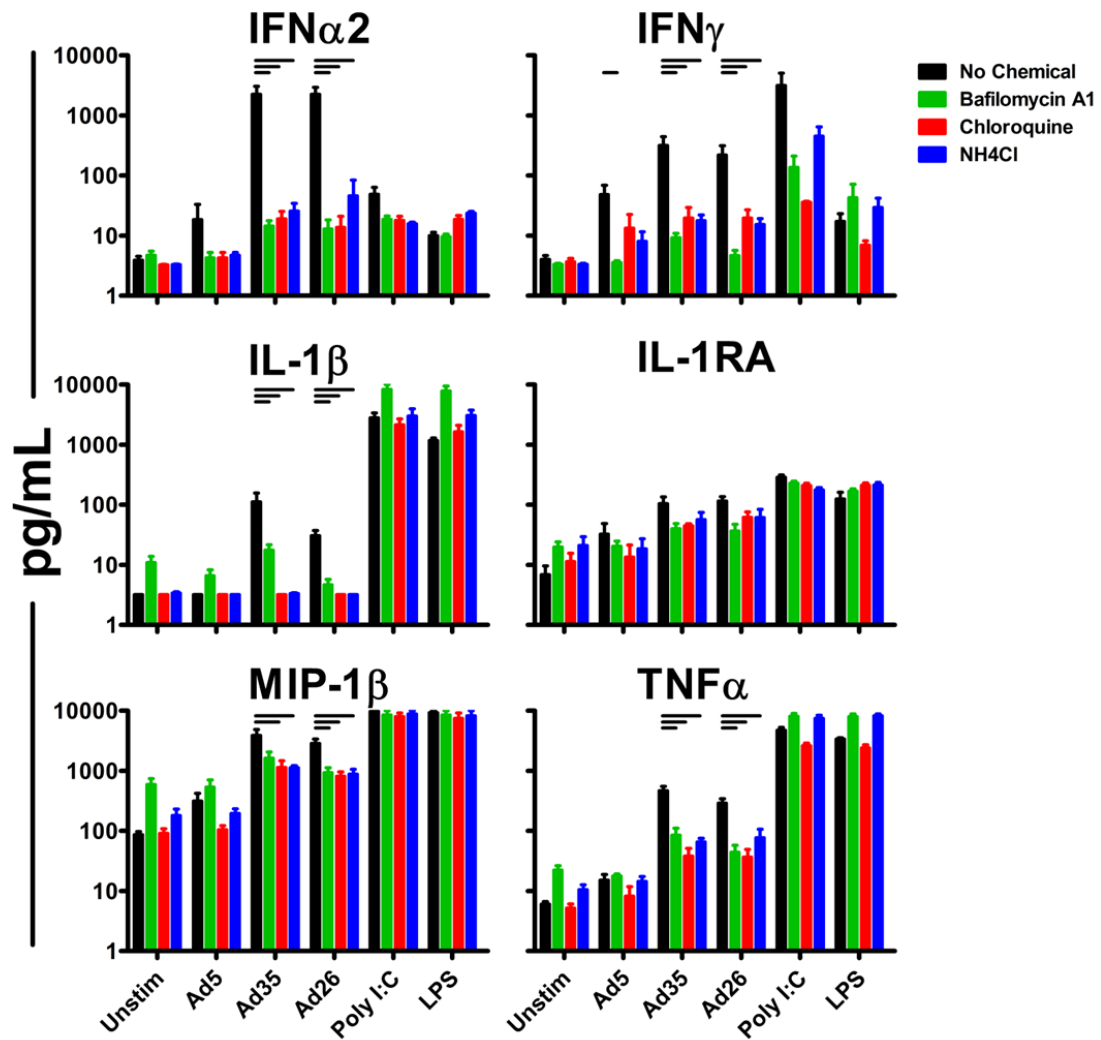


Figure 4.2. Ad innate immune stimulation is blocked by inhibitors of late endosomal acidification.

Fresh human PBMC ($n=4/\text{group}$) were pre-incubated for 1 h with Bafilomycin A1 (BafA) ($1\ \mu\text{M}$), Chloroquine ($200\ \mu\text{M}$), or Ammonium chloride ($1\ \text{mM}$) prior to infection with 1,000 vp/cell of Ad5, Ad35, or Ad26. Induced cytokines and chemokines were measured 24 h post-infection by Luminex assays. Bars indicate $p=0.0286$, Kruskal-Wallis Tests. High Molecular Weight (HMW) Poly I:C (Poly I:C) ($10\ \mu\text{g}/\text{mL}$) and Lipopolysaccharide (LPS) ($1\ \text{ng}/\text{mL}$) included as positive controls.

Innate immune stimulation by Ad5, Ad35, and Ad26 is sensitive to Cathepsin B, Cathepsin L, and Pan-Cathepsin inhibition. We next evaluated the role of specific cathepsins in Ad innate sensing. Cathepsins are a group of late endosomal papain-like proteases which serve several important roles in late endosomal cargo processing as well as autophagy and apoptosis (30). Fresh human PBMC (n=4/group) were pre-incubated for 1h with the Cathepsin B inhibitor Ca074-Me, Cathepsin L inhibitor CAA0225, a Cathepsin G inhibitor, the pan-Caspase inhibitor Z-VAD, the pan-Cathepsin inhibitor Z-FA, or a DMSO vehicle control. Cells were then stimulated with 1,000 vp/cell of Ad5, Ad35, or Ad26. Elicited cytokine and chemokine levels were analyzed 24 h post-infection by Luminex assays as described above.

Human PBMC pre-incubated with Ca074-Me, CAA0225, or Z-FA showed reduced levels of many cytokines and chemokines produced in response to all Ad vectors. In particular, levels of IFN- γ were reduced following pre-incubation of human PBMC with these cathepsin inhibitors in response to Ad5, Ad35, and Ad26 (Figure 4.3). Interestingly, pre-incubation of PBMC with the pan-Caspase inhibitor Z-VAD also reduced levels of elicited MIP-1 β by both Ad35 and Ad26. Pre-incubation with a Cathepsin G inhibitor or DMSO vehicle control had no significant effect on elicited levels of cytokines and chemokines by Ad35 and Ad26 (Figure 4.3). These results indicate that Ad5, Ad35, and Ad26 are all inhibited by these Cathepsin inhibitors, including blockade of Cathepsin B and pan-Cathepsin inhibition, and to a lesser extent Cathepsin L, thus supporting the functional relevance of late endosomal localization of Ad to triggering innate immunity.

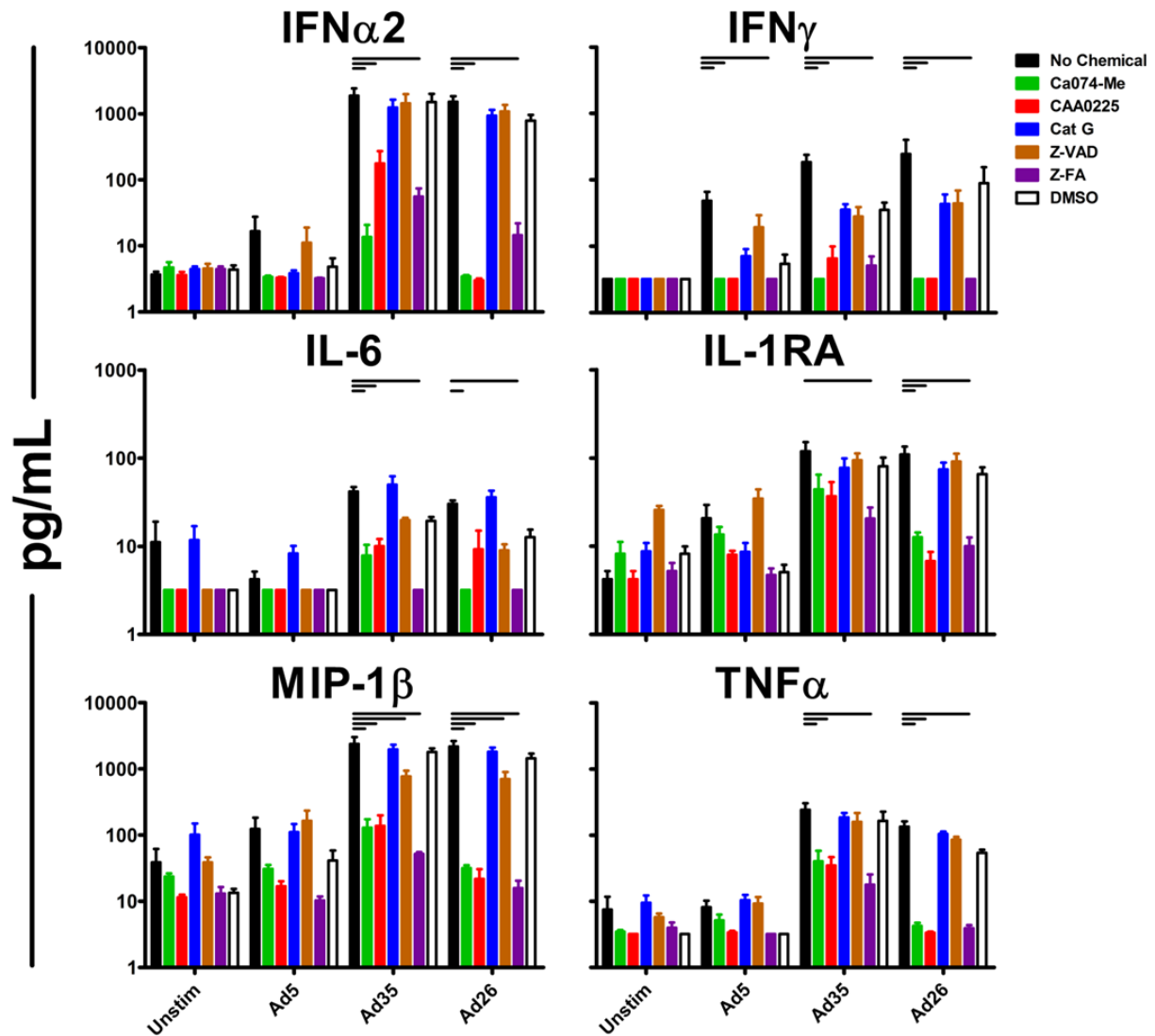


Figure 4.3. Ad innate immune stimulation is sensitive to Cathepsin B, Cathepsin L, and pan-Cathepsin inhibition. Fresh human PBMC (n=4/group) were isolated as described above and pre-incubated for 1 h with the Cathepsin B inhibitor Ca074-Me (10 μ M), the Cathepsin L inhibitor CAA0225 (10 μ M), a Cathepsin G inhibitor (10 μ M), the pan-Caspase inhibitor Z-VAD (100 μ M), the pan-Cathepsin inhibitor Z-FA (100 μ M), or a DMSO vehicle control. Cells were stimulated with 1,000 vp/cell of Ad5, Ad35, or Ad26 and cytokines measured 24h post-infection by Luminex assays. Bars indicate p=0.0286, Kruskal-Wallis Tests.

Inhibition of Caspase-1 reduces innate immune stimulation elicited by Ad5, Ad35, and Ad26. Caspases are proteases that serve as major components of pro-apoptotic and pro-inflammatory pathways (31). To assess the role of caspases in Ad innate stimulation, fresh human PBMC (n=3-4/group) were stimulated with Ad5, Ad35, or Ad26 following pre-incubation for 1 h with the Caspase-9 inhibitor Z-LEHD, the Caspase-1 inhibitor Z-WEHD, the Caspase-4 inhibitor Z-YVAD, or DMSO vehicle control. Pre-incubation of human PBMC with the Caspase-1 inhibitor Z-WEHD reduced elicited levels of IFN- α 2 and IFN- γ elicited by Ad5. Pre-incubation of human PBMC with Z-WEHD also led to a marked reduction of IFN- α 2 and IFN- γ elicited by Ad35 and Ad26 (Figure 4.4). In contrast, pre-incubation of human PBMC with the Caspase-9 inhibitor Z-LEHD or the Caspase-4 inhibitor Z-YVAD had little to no effect on the innate cytokine stimulation by Ad5, Ad35, and Ad26. These data suggest a role for Caspase-1 for Ad5, Ad35, and Ad26 induced innate immune responses.

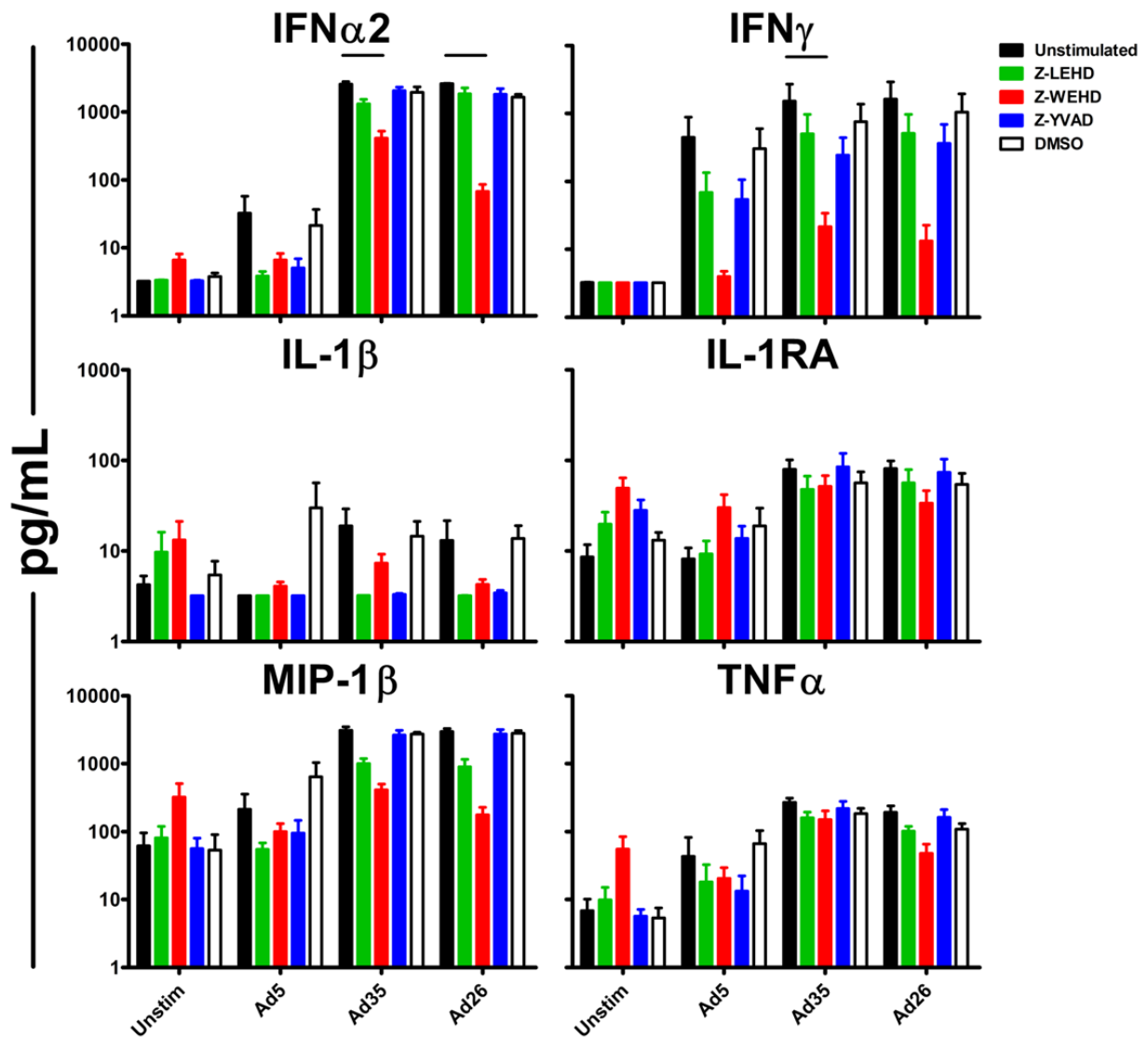


Figure 4.4. Ad innate immune stimulation is reduced by inhibition of Caspase-1. Fresh human PBMC (n=3-4/group) were isolated by the Ficoll-hypaque density gradient method and pre-incubated for 1 h with the Caspase-9 inhibitor Z-LEHD (100 μ M), the Caspase-1 inhibitor Z-WEHD (100 μ M), the Caspase-4 inhibitor Z-YVAD (100 μ M), or DMSO vehicle control. PBMC were then stimulated with 1,000vp/cell of Ad5, Ad35, or Ad26 and elicited cytokine and chemokine levels were measured 24h following infection by Luminex assays. Bars indicate p=0.0286, Kruskal-Wallis tests.

Innate stimulation by Ad5, Ad35, and Ad26 is initiated between 0-6 hours post infection. To determine the timing of the initial triggering of the innate sensing pathways by Ad vectors, fresh human PBMC were stimulated with 1,000 vp/cell of Ad5, Ad35, or Ad26. At 0, 4, 6, 8, or 12 h post-infection, cells were treated with chloroquine, ammonium chloride, Ca074-Me, CAA0225, or Z-FA at the concentrations described above. Cytokine and chemokine levels were assessed 24 h following infection by Luminex assays and compared to infected cells with mock inhibition.

Addition of inhibitors at the time of infection (0 h) effectively inhibited induction of IFN- α 2, IFN- γ , and TNF- α (Figure 4.5), in agreement with the previous experiments (Figures 4.2-4.4). For all three vectors, the majority of inhibition of innate sensing by chloroquine and Z-FA occurred earlier than 6 h post-infection, with the most prominent inhibition caused by Z-FA occurring up to 12 h post-infection (Figure 4.5). Interestingly, inhibition of cytokine responses elicited by Ad26, and to a lesser extent by Ad5, was sensitive to inhibition up to 6 h post infection, while those elicited in response to Ad35 were able to be significantly inhibited up to 4 h post infection. Together these data indicate that innate immune sensing of Ad5, Ad35, and Ad26 was initiated between 0-6 h post-infection. These findings are consistent with the possibility that localization of Ad vectors to the late endosome is important for Ad innate sensing, as inhibition of innate immune sensors reduced Ad innate immune stimulation during times at which Ad vectors were observed to be present in late endosomes.

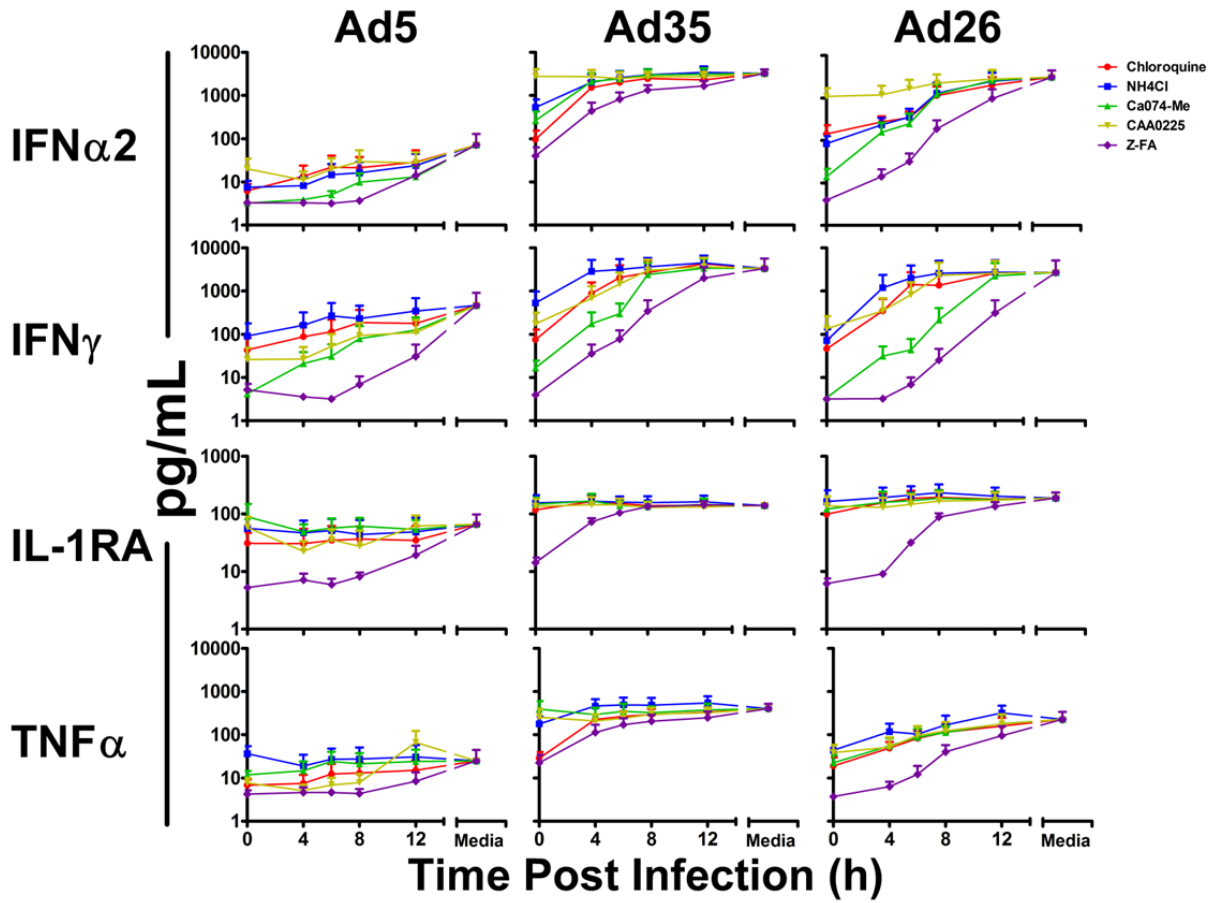


Figure 4.5. Ad innate immune stimulation occurs with similar kinetics and is sensitive to chemical inhibitors from 0-6 hours post-infection. Fresh human PBMC (n=4/group) were isolated as described above and stimulated with 1,000 vp/cell of Ad5, Ad35, or Ad26. Chemical inhibitors Chloroquine, Ammonium chloride, Ca074-Me, CAA0225, or Z-FA were added at the indicated times following infection. Levels of elicited cytokines and chemokines were measured 24 h following infection by Luminex assays and compared to an infected, mock-inhibited control (Media).

Chemical inhibition of innate immune stimulation does not perturb transduction efficiency. We next investigated whether chemical inhibition of Ad innate immune stimulation was simply due to blockade of Ad infectivity. To test whether chemical inhibitors had an effect on Ad35 or Ad26 transduction efficiency, human PBMC (n=4/group) were treated at the time of infection with chloroquine, ammonium chloride, Ca074Me, CAA0225, Z-FA, or DMSO vehicle control as described above. These cells were then stimulated with 1,000 vp/cell of Ad35 or Ad26 expressing eGFP. Levels of elicited cytokines and chemokines were measured 24 h post infection by Luminex assays, and levels of transduced cells were measured by flow cytometry.

Similar to our results above, chloroquine, ammonium chloride, Ca074-Me, CAA0225, and Z-FA reduced cytokine and chemokine induction in human PBMC elicited by Ad35 or Ad26 expressing eGFP at 24 h post infection (data not shown). Flow cytometry on stimulated cells showed no inhibition of transduction of PBMC by either Ad35 or Ad26 for any of the chemical inhibitors analyzed, as measured by %eGFP positive cells 24 h post-infection (Figure 4.6). These results confirm that chemical inhibition of Ad35 and Ad26 innate sensing did not substantially reduce vector infectivity.

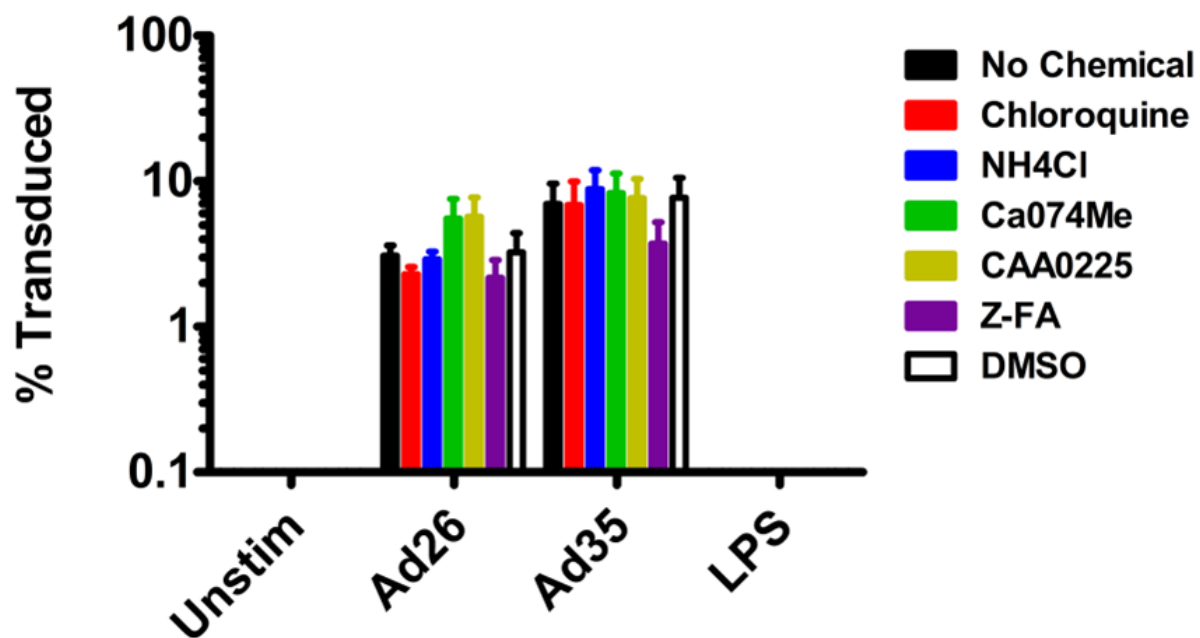


Figure 4.6. Chemical inhibition of Ad5, Ad35, or Ad26 innate immune signaling does not block Ad transduction. Fresh human PBMC (n=4/group) were synchronously inhibited with Chloroquine, Ammonium chloride, Ca074-Me, CAA0225, Z-FA, or DMSO vehicle control at same concentrations as above, and stimulated with 1,000 vp/cell of Ad35 or Ad26 expressing eGFP 24 h following infection percent eGFP cellular transduction was measured by flow cytometry.

Both Ad fiber and capsid components influence intracellular trafficking pathways. Both fiber and capsid components of Ad5 and Ad35 can influence innate stimulatory properties of Ad vectors (12). In particular, both Ad35 fiber and capsid components were required to confer the increased innate stimulatory properties of Ad35 relative to Ad5. We sought to determine the influence of Ad fiber and capsid components on access to the late endosomal compartment and the associated Ad innate sensing mechanisms. To explore the influence of the Ad fiber and capsid on Ad intracellular trafficking, we utilized Ad35k5, a chimeric Ad35 with its fiber knob domain replaced with that of Ad5, and Ad5f35, a chimeric Ad5 with its fiber protein replaced with that of Ad35. A549 cells were infected with Ad35k5 and Ad5f35 vectors and subsequently processed for immunofluorescence histochemistry and confocal microscopy as described in Figure 4.1.

Ad5f35 and Ad35k5 both displayed an initial colocalization with the EEA1 compartment at 30 min (32.9% and 20.2%, respectively) and 60 min (21.4% and 22.4%, respectively), similar to that observed with Ad35. Furthermore, both Ad5f35 and Ad35k5 displayed increased late endosomal trafficking beginning at 2 h post infection, as indicated by higher colocalization relative to Ad5 at 2 h for both LAMP1 (2.8x and 5.9x fold greater colocalization relative to Ad5, respectively; $p=0.0215$ and $p<0.0001$, respectively; Students' t tests) and M6P (5.6x and 8.8x higher colocalization, respectively; $p=0.0003$ and $p<0.0001$, respectively) (Figure 4.7). These data indicate that both fiber and capsid components of Ad vectors influence intracellular trafficking patterns.

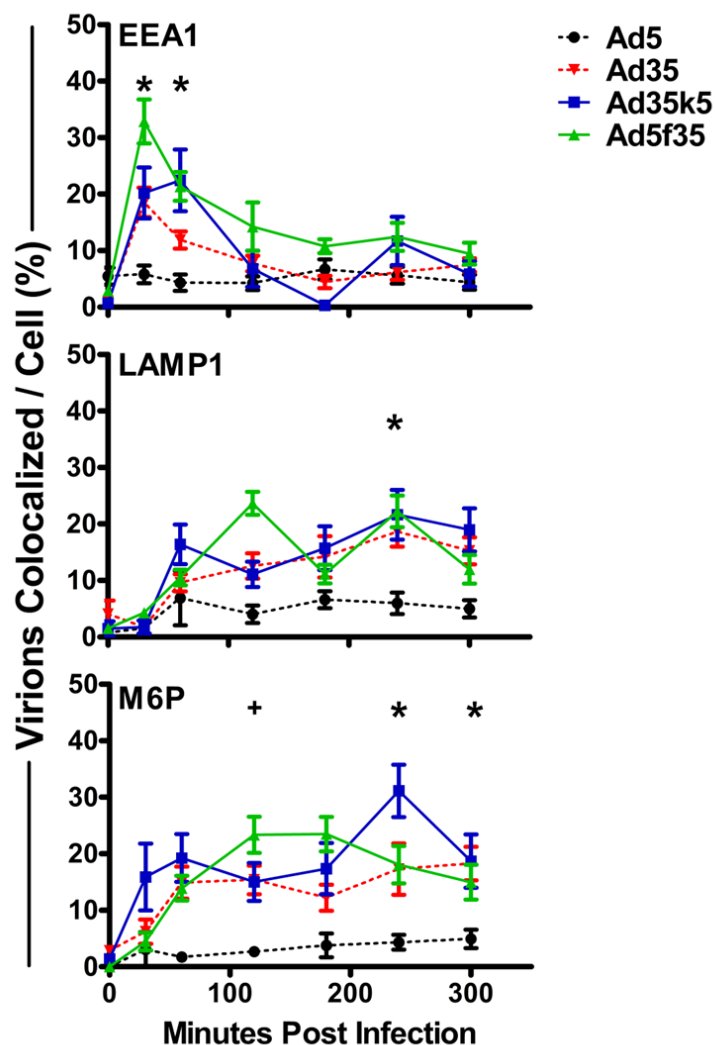


Figure 4.7. Both fiber and capsid components influence Ad vector intracellular trafficking patterns. A549 cells were incubated with 50,000 vp/cell of Atto-633 labeled Ad35k5 or Ad5f35 and fixed at indicated times post infection by immersion in 4% Paraformaldehyde. Cells were stained for the indicated markers by immunohistochemistry and virion colocalization assessed as described above. Graphs indicate virions/cell colocalized with the indicated compartment at indicated timepoints with Ad5 and Ad35 data from Figure 1D shown as dashed lines for reference. Symbols indicate timepoints where both Ad35k5 and Ad5f35 colocalization values were both significantly different than Ad5, with the less significant value indicated (* $p < 0.01$, * $p < 0.001$; Student's t tests).

DISCUSSION

In this study, we show that Ad5, Ad35, and Ad26 differ substantially in their intracellular trafficking patterns, with Ad35 and Ad26 accumulating to a greater degree than Ad5 in late endosomes, and Ad5 rapidly trafficking to the nucleus. All three vectors were sensitive to chemical inhibitors of late endosomal innate immune sensing, in particular endosomal acidification, cathepsin B, and caspase-1. These studies suggest a mechanism that could account for the differential innate immune stimulatory phenotypes of Ad vectors in which intracellular trafficking to the late endosome contributes to these observed differences in innate immunity (12, 13) (Figure 4.8). In the late endosome, virions stimulate stereotypical innate immune pathways that require endosomal acidification, cathepsins, and caspase-1 (Figure 4.8). Taken together, these data suggest that intracellular Ad vector trafficking critically influences innate immune stimulation by various Ad serotypes.

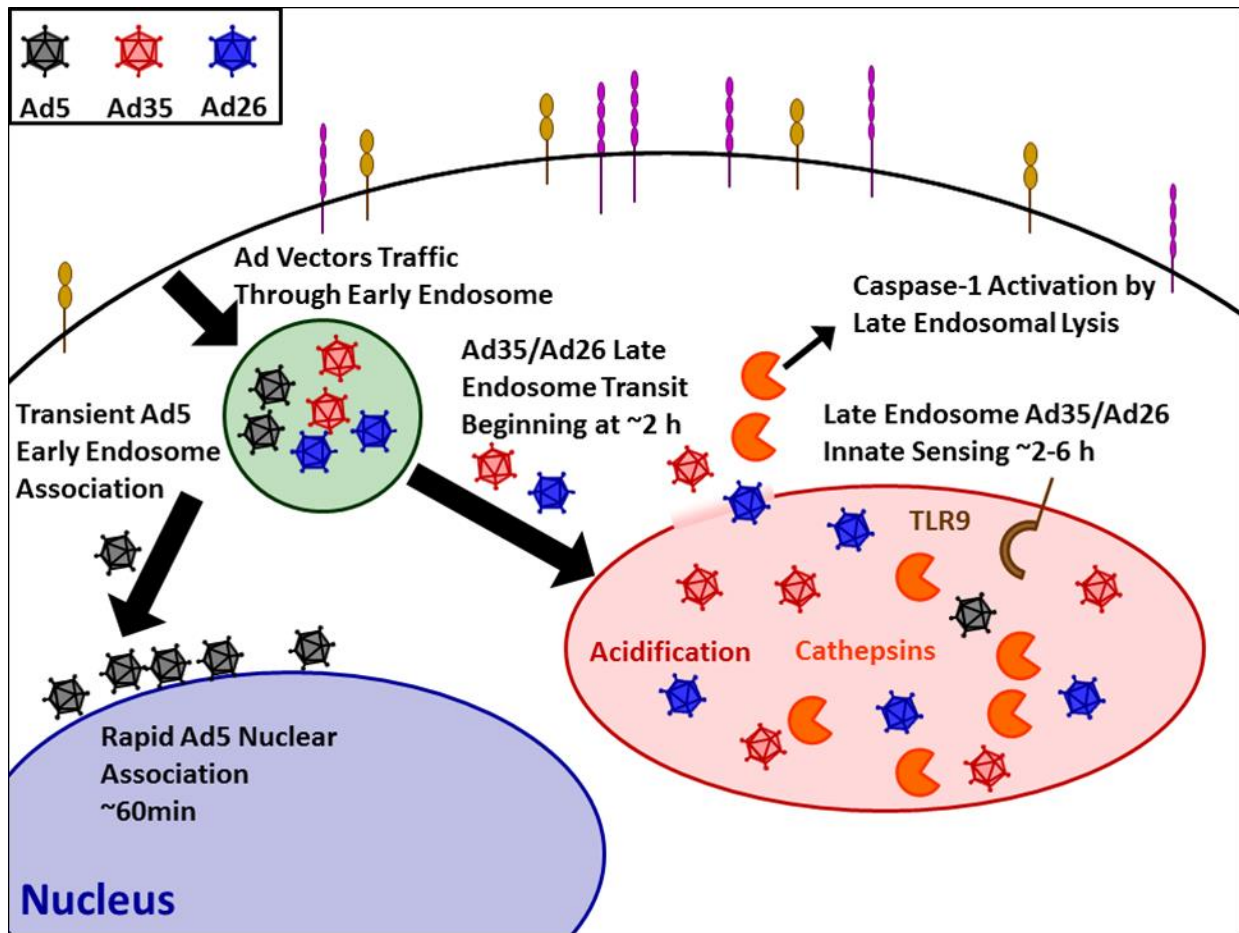


Figure 4.8. Proposed model of differential innate immune stimulation elicited by Ad5, Ad35, and Ad26. Ad5, Ad35, and Ad26 virions enter the cell through an EEA-positive early endosomal compartment. Upon entry to this location, the majority of Ad5 rapidly exits the endocytic pathway into the cytosol where it subsequently traffics to the peri-nuclear space by 60 min post-infection. Ad35 and Ad26, as well as a small subset of Ad5 virions, accumulate in the late endosomal compartment 2-6 h following infection. At approximately 2-6 h post-infection, late endosomal Ad virions exit the late endosome and traffic to the nucleus. Accumulation of Ad virions in a late endosomal compartment as well as their release into the cytosol stimulates both an endosomal acidification-dependent as well as a Cathepsin-dependent sensor.

Innate immune stimulation elicited in response to all three Ad vectors was sensitive to inhibition of acidification, cathepsins, and caspase-1, suggesting that accumulation in late endosomes, rather than differential Pattern Recognition Receptor (PRR) stimulation per se, may be responsible for the observed differences in innate immune phenotypes elicited by these vectors. The requirement of endosomal acidification for innate sensing suggests that late endosomal TLR's may be the endosomal acidification-dependent portion of the sensing pathway (Figures 4.2 & 4.8). For example, TLR9 has been previously implicated in Ad vector dsDNA genome sensing, and TLR9 requires endosomal acidification for its function (14, 32–35). Additionally, late endosomal lysis and Cathepsin B release into the cytosol activates the NALP3 inflammasome, and Cathepsin B activity is required for this process (36). The sensitivity of Ad vector innate stimulation to both the Cathepsin B inhibitor Ca074-Me as well as the pan-Cathepsin inhibitor Z-FA for an extended period following Ad endocytosis temporally is consistent with our observation that Ad35 and Ad26 vectors potentially begin exiting the late endosome at 2-6 hours post-infection (Figures 4.1A & 4.5). Additionally, the fact that Ad vector sensing was sensitive to Cathepsin inhibitors for up to 6 h following infection indicates that a defect in endosomal TLR processing is unlikely to account for the reduced innate immune stimulation observed. Of note, recent studies have suggested the importance of the cytosolic DNA sensor cGAS for innate sensing of Ad5 in murine cells (37). The potential influence of cGAS on Ad innate immune sensing as well as putative differences between Ad5, Ad35, and Ad26 on cGAS stimulation will therefore be of interest for further understanding of Ad innate immune sensing. Our data also confirm prior observations of the importance of acid-dependent innate immune sensors and inflammasome activation, and extends these findings by suggesting that differential trafficking of Ad serotypes to late endosomal compartments contributes to their innate immune stimulatory phenotypes. Importantly, these data also suggest, in agreement with

previous studies showing Ad vector innate sensing is redundant *in vivo*, that several pattern recognition receptors are likely important for Ad vector sensing (13, 16, 38).

Both the fiber as well as other capsid components appear to contribute to the innate stimulatory properties of Ad vectors, suggesting roles of receptor-binding proteins as well as acid-dependent capsid protease components in determining Ad innate immune stimulatory phenotypes (12). Interestingly CD46-binding by fiber protein appears to be sufficient to confer late endosomal trafficking on Ad vectors (22). These data suggest that Ad innate immune stimulation phenotypes observed in previous studies may be reflective of increased late endosomal trafficking conferred by Ad fiber protein, rather than primary receptor usage (15, 17, 34). Furthermore, the increased innate stimulatory capacity of Ad5f35 relative to Ad35k5, despite their roughly equivalent ability to access late endosomal compartments, suggests an active role of CD46 in influencing Ad vector innate immune stimulation, a possibility which warrants further investigation.

In summary, our data suggest a potential mechanism by which Ad5, Ad35, and Ad26 vectors differentially stimulate innate immunity. These vectors trigger similar innate immune pathways, but the extent of stimulation is driven by differential intracellular trafficking to late endosomes, resulting in profound differences in innate immunity. These results have important implications for the development of vectors for vaccines and gene therapy and could lead to strategies for rational vector design that specifically tune desired innate immune phenotypes.

MATERIALS AND METHODS

Viruses. E1/E3-deleted replication incompetent Ad5, Ad35, Ad26, Ad35k5, and Ad5f35 vectors expressing no transgene and eGFP were generated as previously described (39)(1). Briefly, viruses were produced in E1-complimenting PER.55K cells and purified by CsCl gradient ultracentrifugation.

Cells. A549 cells (ATCC) were serially passaged in Dulbecco's Eagle's Modified Medium supplemented with 10% Fetal Calf Serum (FCS) and grown at 37°C, 5% CO₂. Normal human blood was collected in the presence of sodium heparin and PBMC isolated by the Ficoll-hypaque density gradient method (40). Cells were resuspended at a concentration of 1×10^6 cells/mL in R10 medium (RPMI, 10% Fetal Calf Serum (FCS), 50U/mL penicillin, 50µg/mL streptomycin) and further utilized in *in vitro* assays.

Chemicals. The endosomal inhibitor Bafilomycin A1 (Enzo Life Sciences, Farmingdale, NY), Cathepsin inhibitors Ca074-Me, CAA0225, Cathepsin G Inhibitor (Millipore, Billerica, MA), and Z-FA (R&D Systems, Minneapolis, MN), and the Caspase inhibitors Z-LEHD, Z-WEHD, Z-YVAD, and Z-VAD (R&D Systems, Minneapolis, MN) were dissolved in sterile DMSO. Endosomal acidification inhibitors Chloroquine (Sigma-Aldrich, St. Louis, MO), and Ammonium chloride (Sigma-Aldrich, St. Louis, MO) were reconstituted in sterile deionized water. Concentrations of inhibitors utilized were determined from prior literature or by titration for function.

***In Vitro* Stimulation Assay.** For all reactions 1×10^6 PBMC were stimulated with 1,000 vp/cell of the indicated Ad vectors. Cells were treated with various chemical inhibitors prior to or following stimulation with Ad vectors. Cells were cultured at 37°C, 10% CO₂, and culture supernatants were harvested 24 following Ad vector stimulation by centrifugation at 1,400 rpm for 5 minutes. Supernatants were analyzed using the Millipore Milliplex MAP Magnetic Human Cytokine/Chemokine Panel (Millipore, Billerica, MA) according to manufacturer's protocol. Luminex data were acquired on a BioPlex 200 instrument running BioPlex Manager v4.1 (Bio-Rad, Hercules, CA) with an 80% to 120% standard

acceptance range. Data were analyzed using GraphPad Prism v5.0. Means were compared between groups using Kruskal-Wallis Tests and plotted as means and standard error of the mean (S.E.M.).

Fluorescent Labeling of Adenoviral Vectors. Adenoviral vectors were fluorescently labeled with the NHS-ester of Atto633 (Atto-tec GmbH, Siegen, Germany) as previously described (20). Briefly, Ad vectors were incubated for 10-20 minutes at RT with Atto633 and washed 3x at 4°C with ice-cold PBS containing 5% Sucrose (w/v) by centrifugation at 3,000 rpm for 30 min in Amicon 10,000kDa molecular weight centrifuge concentration vials (Millipore, Billerica, MA). Atto633:Capsomere ratio was determined using the extinction coefficient of Atto633 and Abs₂₆₀. All preparations had ratios between 0.5 and 1.8 and displayed no defect in cellular binding or trafficking.

Infection of A549 Cells with Atto633-Labeled Ad Vectors. 15,000 A549 cells were plated onto No.1 German Glass 15mm Coverslips (BD Biosciences, San Jose, CA) and incubated O/N at 37°C, 5% CO₂. Cells were placed on ice, washed 2x with ice-cold Phosphate Buffered Saline (PBS), and overlaid with 5,000-50,000 vp/cell of indicated Atto633-labeled Ad vectors diluted in unsupplemented DMEM. Cells were incubated on ice with Ad vector for 90 min, with gentle rocking every 15 min. Cells were washed 2x with ice-cold PBS and viral infection induced with overlay of 37°C pre-warmed DMEM supplemented with 10% FCS. Cells were cultured at 37°C, 10% CO₂. At indicated timepoints, cells were fixed by immersion into ice-cold 4% Paraformaldehyde diluted in PBS for 10 min. Cells were washed 2x with ice-cold PBS and permeabilized by immersion in 0.01% Saponin diluted in PBS supplemented with 5% FCS for 15min. Cells were washed 3x in ice-cold PBS and stored for further use.

Immunofluorescence Histochemistry. Cells were stained by overlay onto a drop of the indicated antibodies at the indicated concentrations diluted in PBS supplemented with 5% FCS in a humidified chamber protected from light. Primary antibodies were incubated for 1 hour and secondary antibodies were incubated for 30 min. All staining steps were followed by 3x washes with 1 mL ice-cold PBS

supplemented with 5% FCS. Antibodies and concentrations used were Rabbit anti-LAMP1 (1:1000) (Abcam, Cambridge, MA), Mouse anti-EEA1 (1:500) (BD Biosciences, San Jose, CA), Mouse anti M6P (1:100) (Abcam, Cambridge, MA), Goat anti-Mouse IgG (2 μ g/mL), and Donkey anti-Rabbit IgG (2 μ g/mL) (Life Technologies, Grand Island, NY). Following staining, coverslips were inverted onto a drop of ProLong Gold containing DAPI mounting medium (Life Technologies, Grand Island, NY) on microscope slides, were allowed to cure overnight at room temperature, and were stored at -20°C for future use.

Confocal Microscopy. Slides were analyzed at the Beth Israel Deaconess Medical Center Imaging Core on a Zeiss LSM Meta with a 10x optical magnification and a Zeiss 63x oil-immersion lens. Images were acquired using Zeiss LSM 510(Carl Zeiss GmbH, Jena, Germany) in the Z-stack setting with optical slices taken at a width of 0.5 μ m and an interval of 0.3 μ m. For each virus, timepoint, and endosomal marker, at least 3x coverage of 2-7 independent experiments were performed.

Image Processing and Analysis. Images were processed utilizing Volocity Software (Perkin-Elmer, Waltham, MA). Virions were detected as particles of intensity >3x standard deviation of image at least 0.078 μ m³. Endosomal compartments were measured as positive by signal >3x standard deviation of image. Cells were drawn manually as Regions of Interest (ROI) and virion colocalization within ROI's was measured by Manzel correlation coefficient of 0.5 or greater. Virion colocalization with a given compartment was calculated on a per ROI basis as a percentage of total individual ROI virions. Image figures were generated using Fiji package for ImageJ. All images for a given endosomal marker or virus were adjusted identically for brightness and contrast for the purpose of image printing, and images utilized for Volocity analysis were unaltered prior to analysis. Colocalization of viruses with a given compartment was analyzed by GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA), and differences in colocalization between viruses was assessed by Student's t tests.

Data Analysis and Statistics. Cytokine and chemokine levels were assessed from Luminex assays as determined using a 5-parameter logistic model. Levels of cytokines and chemokines for inhibitor treated and untreated cells was assessed by Kruskal-Wallis tests. Colocalized virions/cell was calculated for each endosomal marker and virus analyzed and colocalization amounts were compared between viruses by Student's t tests.

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**Chapter 5: The Canarypox Vector ALVAC Induces Distinct Cytokine Responses Compared to the
Vaccinia-Based Vectors MVA and NYVAC in Rhesus Monkeys**

This chapter is based on the peer-reviewed publication:

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INTRODUCTION

Poxviral vectors are utilized for vaccination against multiple pathogens, including HIV-1 and cancer (1–3). A recent study utilizing the canarypox-based vector ALVAC with an HIV-1 gp120 protein boost showed 31.2% efficacy against HIV-1 infection in a Phase III trial in Thailand (RV144) (4). While analysis of data from the RV144 trial has indicated several possible correlates of vaccine-induced protection (5), the innate immune stimulatory properties of ALVAC vectors and how they might influence adaptive immune responses remain poorly described. In this study, we compared innate immune stimulation as measured by serum cytokine and chemokine levels following vaccination of rhesus monkeys with ALVAC and two vaccinia-based vectors Modified Vaccinia Ankara (MVA) (6) and New York Vaccinia Virus (NYVAC)(7).

RESULTS

We initiated studies by assessing serum cytokine and chemokine levels following vaccination with replication-incompetent ALVAC, MVA, or NYVAC vectors in 32 rhesus monkeys. Rhesus monkeys (*Macaca mulatta*) (n=8-12/group) were injected intramuscularly (i.m.) with 1×10^8 pfu of ALVAC, MVA, or NYVAC vectors expressing SIV Gag, Pol, and Env (8–10). All vectors used in these studies displayed similar purity and infectivity. Sera were collected on days 0, 1, 3, 7 and 14 following vaccination. Systemic cytokine and chemokine levels were assessed by Luminex and ELISA analyses as previously described (11). Longitudinal analysis of systemic cytokine and chemokine levels following ALVAC vaccination revealed potent but transient induction of proinflammatory cytokines and chemokines on day 1 post-immunization (Figures 5.1A & 5.1B). In contrast, lower induction of proinflammatory cytokines and chemokines was observed in monkeys vaccinated with MVA or NYVAC (Figures 5.1A & 5.1B). Animals that received ALVAC displayed greater fold induction over averaged group baseline than those that received MVA or NYVAC for interleukin 1 beta (IL-1 β) (411- and 408-fold greater induction, respectively; $p=0.0002$, $p<0.0001$, respectively; Mann-Whitney U Test), interleukin 6 (IL-6) (83- and 6.3-fold greater induction, respectively; $p=0.0003$, $p=0.0043$, respectively), tumor necrosis factor alpha (TNF- α) (48- and 29-fold greater induction, respectively; $p=0.0002$ and $p<0.0001$, respectively), monocyte chemotactic protein-1 (MCP-1) (4- and 3-fold greater induction, respectively; $p=0.0002$ and $p<0.0001$, respectively), macrophage inflammatory protein alpha (MIP-1 α) (37- and 79-fold greater induction, respectively; $p=0.003$ and $p=0.0004$, respectively), and macrophage inflammatory protein beta (MIP-1 β) (9- and 10-fold greater induction, respectively; $p=0.0002$ and $p<0.0001$) at d1 following vaccination. These data show that ALVAC induced a distinct proinflammatory response compared to MVA and NYVAC in rhesus monkeys.

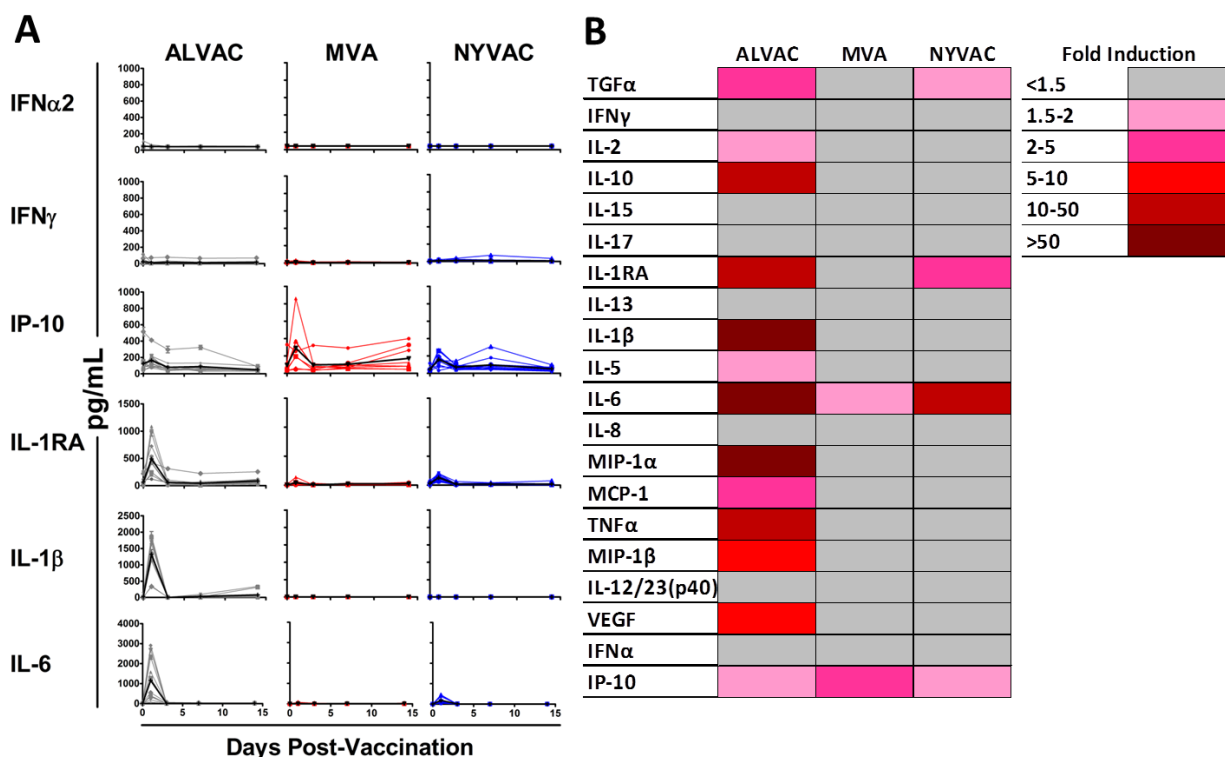


Figure 5.1. Serum concentrations of cytokines and chemokines in rhesus monkeys following poxvirus vector vaccination. Rhesus monkeys (n=8-12/group) were inoculated i.m. with 1×10^8 pfu of ALVAC, MVA, or NYVAC expressing SIV Gag, Pol, and Env. Sera were collected on days 0, 1, 3, 7 and 14 following vaccination, and systemic levels of cytokines and chemokines were analyzed by Luminex and ELISA assays. (A) Systemic levels of selected cytokines and chemokines following vaccination with the various poxvirus vectors, with individual levels shown as colored lines and group mean over time shown with black lines. (B) Mean fold induction for all cytokines and chemokines at day 1 post vaccination with poxvirus vectors in rhesus monkeys. Values shown are group mean fold induction over the averaged group baseline.

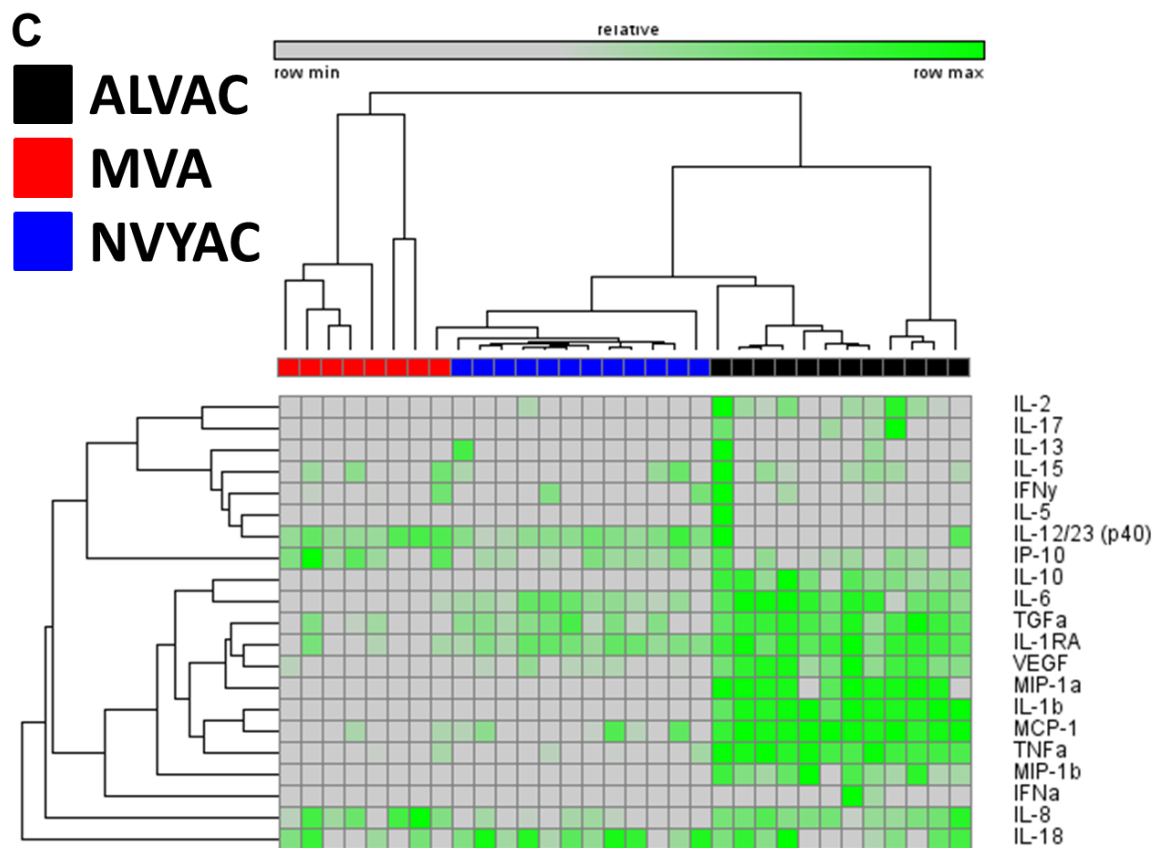


Figure 5.1., continued. (C) Cytokine fold induction over grouped baseline for ALVAC, MVA, and NVYAC vaccinated rhesus monkeys following vaccination. Monkeys are clustered in an unsupervised hierarchical analysis based upon individual fold induction over baseline values for all cytokines. Clustering analysis performed in Gene-E. Values indicate log individual fold induction over grouped baseline of indicated cytokines and chemokines at day 1 following vaccination with the various poxvirus vectors.

Unsupervised hierarchical clustering of animals based upon fold induction at day 1 post-vaccination over individual baseline for all cytokines and chemokines analyzed indicated further that responses elicited by ALVAC, MVA, and NYVAC are all distinct from one another (Figure 5.1C). ALVAC induced a distinct proinflammatory cytokine milieu, but differences were also observed between MVA and NYVAC for several cytokines such as IL-6, transforming growth factor alpha (TGF- α), TNF- α , and MIP-1 α , MIP-1 β (Figure 5.1C). Together, these data indicate that ALVAC, MVA, and NYVAC all induce qualitatively distinct cytokine profiles following vaccination.

We next analyzed the impact of priming with a heterologous vector on elicited cytokines following boosting with ALVAC, MVA, or NYVAC. Rhesus monkeys (n=12/group) were injected intramuscularly (i.m.) with 3×10^{10} vp adenovirus serotype 26 (Ad26) vector expressing SIV Gag, Pol, and Env. Twenty-four weeks following priming vaccination, animals were boosted i.m. with 1×10^8 pfu ALVAC, MVA, or NYVAC expressing SIV Gag, Pol, and Env. Longitudinal analysis of serum cytokines and chemokines following the boost immunization with ALVAC, MVA, or NYVAC revealed clear induction of proinflammatory and antiviral cytokines such as IL-1 β , IL-6, gamma interferon (IFN- γ), and interferon gamma-induced protein 10 (IP-10), on day 1 following the boost (Figure 5.2A). However, cytokine levels elicited following boosting were lower than those observed following priming for all the vectors, and particularly for ALVAC (Figures 5.1A & 5.2A). Analysis of average fold induction over group average baseline indicated that boosting with ALVAC, MVA, or NYVAC induced comparable cytokine profiles, characterized by IL-1 β , IL-6, IL-1RA, IFN- γ , and IP-10 (Figure 5.2B). These data suggest that Ad26 priming attenuated innate cytokine induction as measured by serum cytokine profiles of ALVAC, MVA, or NYVAC boosted animals. We suspect this may be related to enhanced vector clearance as a result of the adaptive immune responses elicited by the priming immunization, though other factors may play a role.

Furthermore, this highlights the unique proinflammatory response elicited following ALVAC priming immunization.

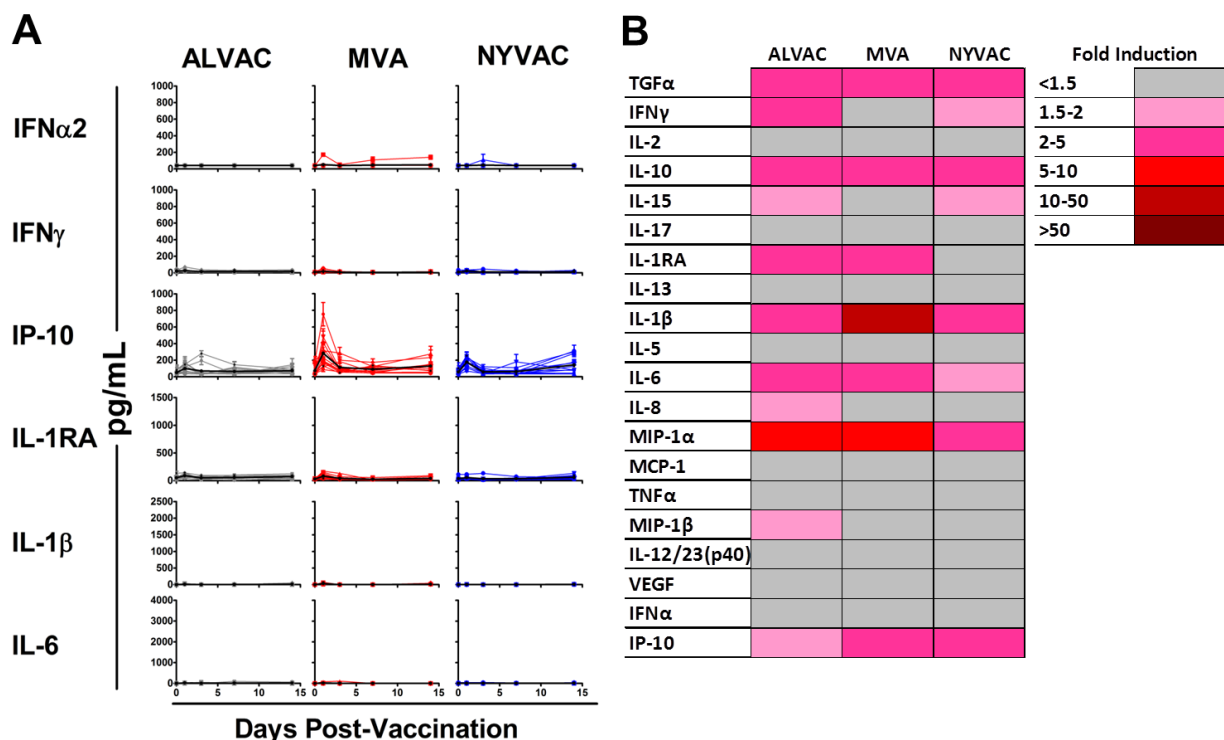


Figure 5.2. Serum concentrations of cytokines and chemokines in rhesus monkeys following poxvirus vector boosting. Rhesus monkeys (n=12/group) were primed im with 3×10^{10} vp Ad26 expressing SIV Gag, Pol, and Env and then boosted i.m. with 1×10^8 pfu of ALVAC, MVA, or NYVAC expressing transgenes. Sera were collected on days 0, 1, 3, 7 and 14 following vaccination and systemic levels of cytokines and chemokines were analyzed by Luminex and ELISA assays. (A) Systemic levels of selected cytokines and chemokines following boosting with the various poxvirus vectors, with individual levels shown as colored lines and group mean over time shown with black lines. (B) Mean fold induction for all cytokines and chemokines at day 1 post boosting with poxvirus vectors in rhesus monkeys. Values shown are group mean fold induction over the averaged group baseline prior to boosting by the various poxvirus vectors.

We next sought to assess cytokine and chemokine induction by ALVAC, MVA, and NYVAC *in vitro* in freshly isolated human PBMC. Fresh human PBMC (n=4/group) were isolated by ficoll-hypaque density gradient centrifugation (12). In order to include all possible cell types shown to be infectable by poxviral vectors, whole PBMC were infected with ALVAC, MVA, or NYVAC vectors at a multiplicity of infection (MOI) of 10 pfu/cell (13). Culture supernatant was analyzed for cytokine and chemokine levels 24 hours post infection by Luminex assays. Similar to our *in vivo* results, stimulation of human PBMC with ALVAC resulted in higher levels of proinflammatory and interferon-related antiviral cytokines and chemokines than with MVA or NYVAC (Figures 5.3A & 5.3B). Stimulation of human PBMC with ALVAC elicited greater levels than with MVA or NYVAC for IFN- γ (41- and 334-fold greater levels, respectively; p=0.0286, both; Mann-Whitney U Test), IL-1 β (37- and 315-fold greater levels, respectively; p=0.0286, both), IL-6 (3- and 15-fold greater levels, respectively; p=0.0571 and p=0.0286, respectively), and TNF- α (7- and 17-fold greater levels, respectively; p=0.0286, both). These *in vitro* results are comparable with our *in vivo* findings with a few notable exceptions, such as a lack of difference observed in the elicitation of IFN- α 2 *in vitro*.

We sought to assess the contribution of various PBMC subsets, some of which previously shown to be readily transduced by Vaccinia virus, to the production of cytokines and chemokines elicited by the ALVAC, MVA, and NYVAC vectors (13). Human PBMC (n=2-6/group) were isolated as described above and depleted of T cells, monocytes and macrophages (MonoMac), myeloid dendritic cells (mDC), or plasmacytoid dendritic cells (pDC) by magnetic separation as previously described (11). Depletion was confirmed by flow cytometry (data not shown). Cells were then infected with an MOI of 10 pfu/cell of ALVAC, MVA, or NYVAC. Supernatants were analyzed 24 hours post infection for elicited cytokines and chemokines by Luminex analyses. Depletion of plasmacytoid dendritic cells (pDC) led to a marked reduction in elicited levels of IFN- α 2 (16-fold lower induction; p=0.0022) elicited in response to ALVAC

stimulation relative to unseparated cells (Figure 5.4). Depletion of monocytes and macrophages (MonoMac) resulted in reduced induction of IL-1 β and TNF- α (117- and 37-fold lower induction; $p=0.0043$, both) in response to ALVAC stimulation. Depletion of T cells markedly decreased levels of elicited IFN- γ (15-fold lower induction; $p=0.0087$) upon stimulation with ALVAC. A similar trend was observed with lowered levels of elicited IFN- γ in response to MVA or NYVAC stimulation in PBMC's depleted T cells prior to stimulation (Figure 5.4). Interestingly, depletion of single PBMC subsets was not observed to dramatically alter elicited levels of IFN- $\alpha 2$ or TNF- α in response to MVA or NYVAC stimulation. This result suggests that there may be redundancy of PBMC subsets in the production of these cytokines in response to MVA or NYVAC vectors. Together these results indicate that multiple PBMC subsets likely contribute to the overall cytokine milieu elicited by ALVAC, MVA, and NYVAC vectors.

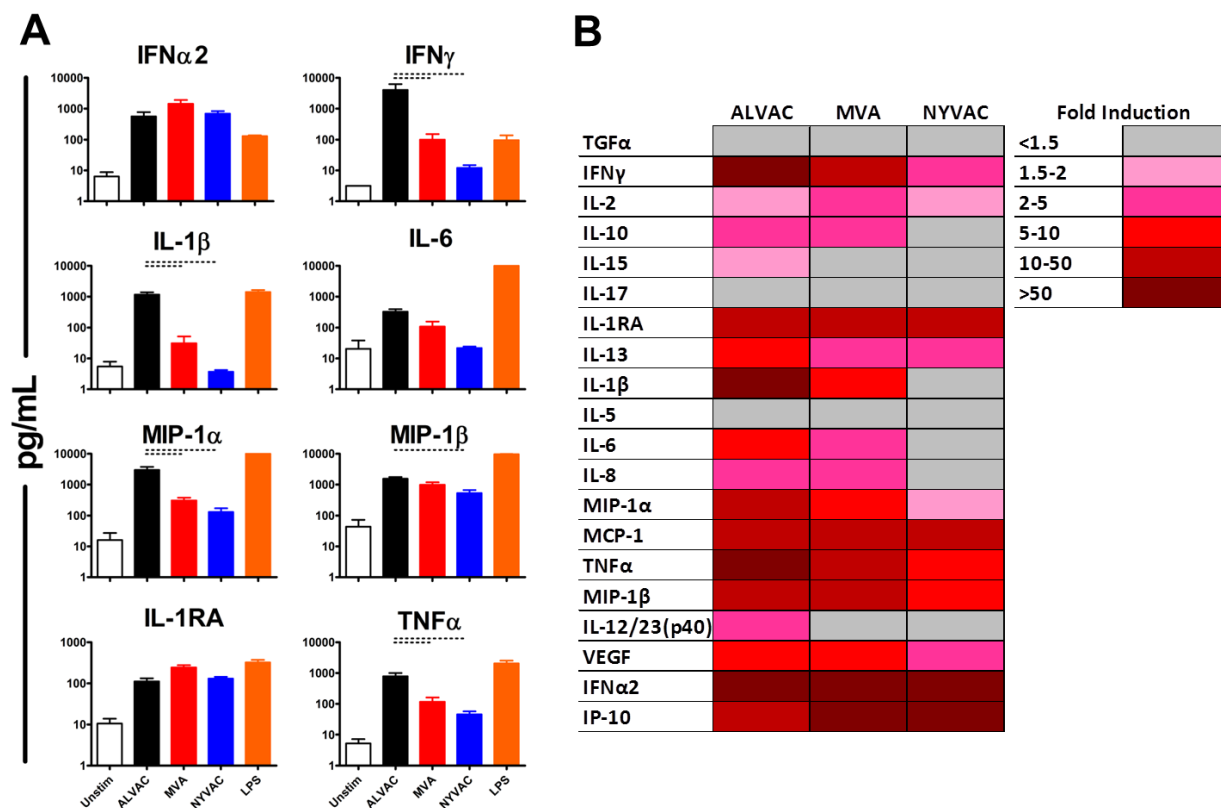


Figure 5.3. Induction of cytokines and chemokines by poxvirus vector stimulation of human PBMC. (A) Fresh whole human PBMC (n=4/group) were stimulated with an MOI=10 pfu/cell of ALVAC, MVA, or NYVAC vectors expressing no transgene. Cytokine and chemokines were measured 24h post infection by Luminex assays. LPS (1ng/mL) included as the positive control. Data are mean and SEM. Bars indicate p values of <0.05 by Kruskal-Wallis Tests. (B) Mean fold induction for all cytokines and chemokines at 24 hours post infection with poxvirus vectors in human PBMC. Values shown are group mean fold induction over the averaged group baseline of unstimulated PBMC control.

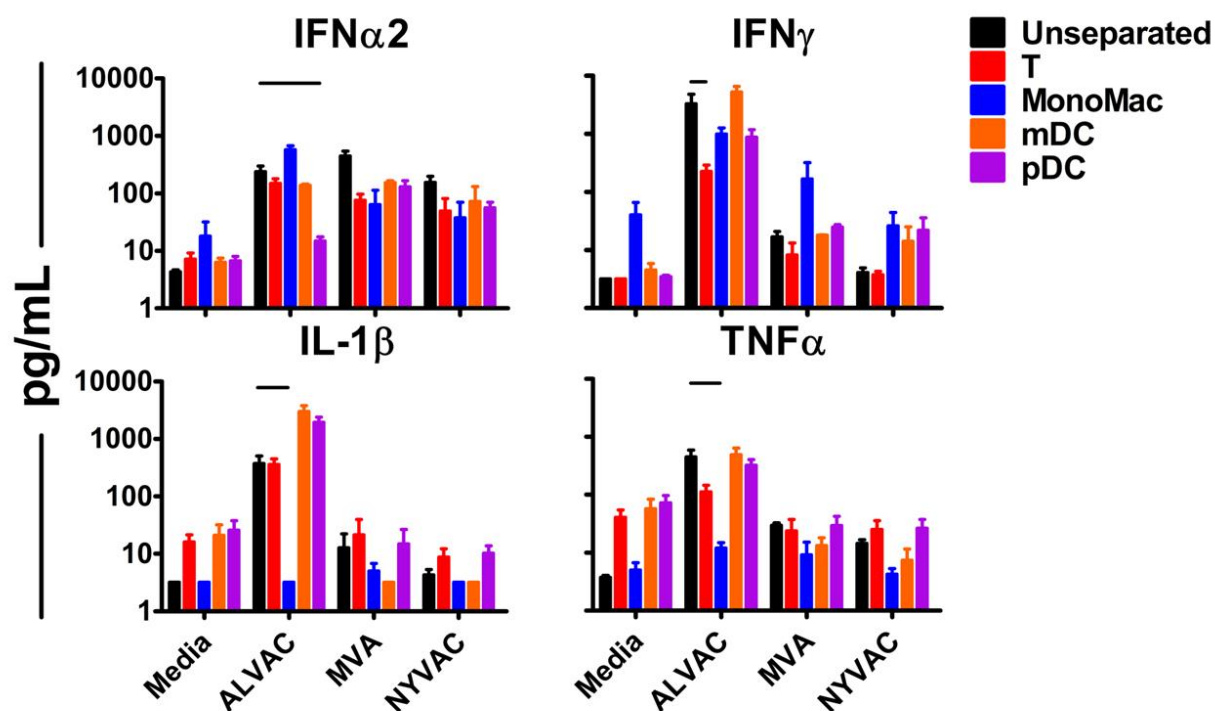


Figure 5.4. Cytokine and chemokine responses elicited by ALVAC in human PBMC depleted of various cell populations. Fresh whole human PBMC (n=2-6/group) were depleted of the indicated PBMC populations and depletion confirmed by flow cytometry (data not shown). Unseparated and depleted cell populations were stimulated with MOI=10 pfu/cell of ALVAC, MVA, or NYVAC and cytokine and chemokine responses were measured 24h post infection by Luminex assays. Data shown as means and SEM. Lines indicate p<0.01 by Kruskal-Wallis tests.

DISCUSSION

In this study, we show that innate immune profiles elicited by ALVAC, MVA, and NYVAC are all distinct. ALVAC elicited a qualitatively and quantitatively different innate immune response compared to MVA and NYVAC both *in vivo* in rhesus monkeys and *in vitro* in human PBMC, characterized by a distinct induction of proinflammatory and interferon-related antiviral cytokines and chemokines. Moreover, MVA and NYVAC also proved different, although these differences were less striking. The stimulatory phenotypes observed following priming with ALVAC, MVA, or NYVAC were reduced when these pox vectors were utilized as a boost. Furthermore, ALVAC's distinct stimulatory phenotype was influenced by several PBMC subsets such as T cells, monocytes and macrophages, and plasmacytoid dendritic cells. These data suggest potentially important biological differences among these three clinically relevant poxvirus vectors. Additional studies will be required to evaluate the correlation between these different innate signatures and subsequent adaptive immune responses and protective efficacy.

A possible source of the observed differences in innate stimulatory phenotypes among ALVAC, MVA, and NYVAC involves their different arsenals of immune regulatory genes. Poxviruses possess a wide array of proteins which serve to block host anti-viral immune responses (14–18). Importantly, ALVAC differs significantly from both Vaccinia virus-derived vectors MVA and NYVAC in terms of extensive phylogenetic divergence as well as genome size (approx. 365kbp vs. approx. 178kbp, respectively) (14, 19) and number of ORF's (7, 19, 20). As stated above, ALVAC, MVA, and NYVAC vectors are replication incompetent when used *in vivo*, and many of ALVAC's ORF's may not be functional in mammalian cells, as evidenced by Avipoxviruses' inability to replicate in mammalian cells (21). Previous reports have indicated increased innate immune gene upregulation following MVA and NYVAC infection relative to vaccinia virus Western Reserve (VV-WR) infection in HeLa cells (22–24), highlighting that attenuation of these vectors by removal of viral immune regulatory genes confers a

stronger innate stimulatory phenotype. These studies reported that MVA elicited a stronger interferon stimulatory phenotype and NYVAC induced a more proinflammatory phenotype, congruent with our results in the present study. Differences in immune gene upregulation were additionally seen following MVA and NYVAC infection in monocyte-derived dendritic cell populations (25). Further studies showed increased innate stimulation by these vectors following expanded deletion of their immune regulatory gene repertoire, highlighting the role of immune gene repertoire of poxvirus vectors on their innate stimulatory phenotypes (26–28). However, far less is known about the innate immune profile of ALVAC and its immune regulatory genes. Our findings suggest that ALVAC stimulation of innate immunity requires multiple several PBMC cellular subsets, which is consistent with prior studies that have suggested that monocyte tropism to be a key difference in innate triggering by ALVAC, MVA, and NYVAC (29). The unique innate immune profile of ALVAC may also relate to its avian origin, as opposed to MVA and NYVAC. The molecular basis of this innate stimulatory phenotype and whether it is unique to ALVAC or extends to other Avipoxviruses warrants further study.

Our results indicate that innate immune profiles elicited by the leading poxviral vaccine vectors are different, suggesting potentially important biological differences. In particular, ALVAC induced a unique proinflammatory cytokine and chemokine response following vaccination in rhesus monkeys and infection in human PBMC. The extent to which these properties are advantageous to vaccines, however, remains to be determined. We previously reported substantial differences in innate immune profiles among various serotypes of adenovirus vectors (11). Taken together, these data suggest that vectors from the same family can differ markedly in their biological and innate stimulatory properties, which may potentially impact the resultant adaptive immune responses and protective efficacy of vector-based vaccines.

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Chapter 6: Conclusions

Viral Vectors Differ Substantially in Their Innate Stimulatory Phenotypes

The results from these studies show that viral vectors differ significantly from one another in their innate stimulatory phenotypes. Importantly, substantial differences in innate stimulatory cytokine and chemokine elicitation were observed not only between Adenovirus (Ad) and Poxvirus (Pox) vector classes, but also marked differences in innate stimulatory properties were observed between members within each class. The observation that viral vectors differ significantly in their innate stimulatory properties both between and within viral families further suggests that viral vectors may possess an ability to influence resultant vaccine-elicited adaptive immune phenotypes through formation of different innate immune stimulatory milieus. Therefore, rather than being passive shuttles used to stimulate adaptive immune responses against a heterologous antigen, vaccine vectors may play an active role in the determination of vaccine-elicited immune responses and protection. While distinct connections between elicited innate and adaptive immune responses induced by vaccines remain unclear, it is possible to hypothesize putative connections between them by analyzing vaccine vectors in broader groupings based upon their cytokine and chemokine elicitation profiles. Ad and Pox vectored vaccines are well described in their adaptive immune phenotypes and broad differences in their observed adaptive immune phenotypes in previous studies may be explained in part by the differences in innate immune cytokine and chemokine responses elicited by their particular vectors (1–5).

Examination of cytokine and chemokine profiles elicited by Ad and Pox vectors in the studies above suggests at least three broad categories of innate immune cytokine and chemokine responses were elicited following vaccination of rhesus monkeys *in vivo* or stimulation of human PBMC *in vitro* in these studies. The first category primarily consists of the low elicitation of cytokines and chemokines elicited by Ad5 and the chimeric vector Ad5HVR48. Following vaccination in rhesus monkeys, Ad5 and Ad5HVR48 failed to induce systemic peaks of most cytokines analyzed at day 1 following vaccination,

the common vector-induced cytokine peak time point observed in these studies (6). However, as revealed by *in vitro* studies in Human PBMC, Ad5 and Ad5HVR48 are both able to elicit both IFN- α 2 and IFN- γ to a relatively low level. However, these vectors were largely unable to stimulate detectable release of proinflammatory markers such as IL-1 β and TNF- α *in vitro* indicating that these vectors either do not induce their expression, or that the producing cell population is absent to a functional degree in whole PBMC. The low levels of cytokines and chemokines commonly elicited by Ad5 and Ad5HVR48 *in vivo* following vaccination relative to those observed *in vitro* first suggests that systemic cytokine levels in the periphery following vaccination reflect “spill over” from a localized reaction within the body, an observation which is further seen with the other viral vectors analyzed. Whether this localized immunostimulatory environment occurs at the site of vaccination, draining lymph node, or potentially other locations remains to be determined. Secondly, the differences observed between the *in vivo* and *in vitro* model systems for Ad5 and Ad5HVR48 suggests that these vectors may elicit relatively little cytokines even in a localized reaction following vaccination. The observed low induction of innate immune cytokines and chemokines following vaccination in rhesus monkeys or human PBMC stimulation is additionally intriguing given observation by several groups that Ad5 and Ad5HVR48 are able to elicit robust CD8⁺ T-cell responses following immunization in mice, non-human primates, and humans (1, 3, 7, 8). The low level of cytokines induced by Ad5 vector are additionally surprising in light of previous observations of enhanced inflammatory responses following direct IV administration in mice and following intramuscular immunization in humans, yet may reflect a dose and/or route dependence for Ad vector innate immune cytokine and chemokine elicitation (9–13). Furthermore, these results suggest that robust cytotoxic CD8⁺ T cell responses may not necessarily predict innate immune cytokine and chemokine elicitation, and vice versa. Results from these studies with Ad5 stimulation therefore suggest that while Ad5 is able to potently induce CD8⁺ T-cell responses *in vivo*, it does not elicit a

correspondingly strong innate immune cytokine and chemokine signature, which may influence elicited cell phenotypes.

The second broad class of innate immune stimulation observed in these studies was elicited by the CD46-utilizing vectors Ad35, Ad26, and Ad48. The innate cytokine and chemokine profiles elicited by these vectors were characterized by increased stimulation of IFN- α 2 and IFN- γ and downstream chemokines such as MCP-1, IP-10, and I-TAC relative to Ad5 and Ad5HVR48, with an additional cytokine and chemokine module consisting of several proinflammatory cytokines such as IL-1 β , IL-1RA, and TNF- α (6). Interestingly, the elicited *in vitro* levels of proinflammatory cytokines and chemokines in response to stimulation with the CD46 Ads was lower relative to the robust induction of IFN- α 2 and IFN- γ elicited by these vectors, highlighting that the innate cytokine and chemokine response elicited by these vectors is primarily interferon-based, a phenotype classically defined as “anti-viral”. This suggests that the levels of cytokines elicited by the CD46 Ads are not uniformly upregulated and that levels of anti-viral and proinflammatory cytokines are not directly correlated. While the CD46 Ad’s elicit equivalent or slightly lower magnitude CD8⁺ T cell responses relative to Ad5, the distinct adaptive immune phenotypes such as increased CD8⁺ T-cell polyfunctionality and enhanced anamnestic potential elicited by Ad35 and Ad26, relative to Ad5 additionally suggest that elicited cytokines and chemokines may influence T cell phenotype rather than response magnitude (1, 3, 14, 15). Further studies to assess whether cytokines and chemokines elicited by the CD46-utilizing Ad vectors have a direct role in shaping such responses in cytotoxic CD8⁺ T cells as well influences on other immune cell types will be of great interest.

The third class of innate immune stimulation observed in these studies is the unique robust proinflammatory cytokine milieu elicited both *in vivo* following priming vaccination in rhesus monkeys and *in vitro* following stimulation of human PBMC with the Canarypox-based vector ALVAC. The unique cytokine signature elicited by ALVAC vaccination displayed the greatest extent of proinflammatory

cytokines of any of the vectors tested (6, 16). Interestingly, antiviral cytokine and chemokine elicitation such as IFN- α 2 and IFN- γ was similar for all three poxviral vectors analyzed, suggesting that the unique stimulatory milieu of ALVAC is primarily proinflammatory in nature. Several possible mechanisms could underlie the potent proinflammatory nature of ALVAC such as tropism differences for monocytes and macrophages or security protein arsenal activity of avipoxvirus vectors versus those of the Vaccinia virus-based vectors MVA and NYVAC (17–23). The mechanism by which this proinflammatory milieu is induced and its influence on possibly unique subsequent adaptive immune response formation is important in light of the protective efficacy shown in the RV144 trial (24). Furthermore, the whether this inflammatory response can be attenuated by inclusion of functional security proteins, perhaps from Vaccinia virus, may further optimize this vector for vaccination applications, and warrants further research.

The innate cytokine and chemokine stimulatory phenotypes of the Pox vectors were observed to be distinct from one another. However, characteristics of the innate immune cytokine and chemokine profiles elicited by the Vaccinia-based vectors MVA and NYVAC enable them to be clustered with groupings described above. As noted in these studies as well as in the literature, MVA induces a primarily interferon-based cytokine response following vaccination in rhesus monkeys or stimulation of human PBMC (16, 25). When unsupervised clustering is performed of *in vivo* cytokine responses following vaccination of rhesus monkeys, the similarity of this response to the highly anti-viral milieu induced following CD46-utilizing Ad vector vaccination is made apparent. Similarly, NYVAC has been observed both previously by other groups as well as in these studies to induce a cytokine and chemokine response skewed toward proinflammatory responses (16, 26, 27). This response, while less robust, resembles in part the potent elicitation of proinflammatory cytokines and chemokines by ALVAC. This similarity is additionally shown in unsupervised clustering of cytokine and chemokine responses

following vaccination in rhesus monkeys. Interestingly, when a similar clustering is performed on cytokine and chemokine responses elicited in human PBMC in response to the various Ad and Pox vectors, Ad5/Ad5HVR48, CD46-utilizing Ads, and ALVAC remain distinct. However, MVA and NYVAC cluster with one another, and together cluster with ALVAC as a separate branch from Ad vectors. This result indicates that the localized reactions to MVA and NYVAC at the site of vaccination or a draining lymph node may be similar, and that alterations in systemic cytokine and chemokine responses to them may reflect broader skewing external to the site of induction by security protein arsenals. The influence of security proteins which differ between MVA and NYVAC on resultant innate and adaptive immune responses will be of interest for further optimization and adaptation of these vectors.

The distinct cytokine responses elicited *in vivo* and *in vitro* by Pox vectors relative to those elicited by either Ad5 or the CD46-utilizing vectors Ad35, Ad26, or Ad48 suggest that the quality of innate immune stimulation elicited by Pox vectors is distinct from those elicited by the Ad vectors. This observation is somewhat unexpected given the extensive differences in viral biology and structure between Adenoviruses and Poxviruses. The observation that innate immune cytokine and chemokine phenotypes differ not only within Ad and Pox vectors, but between them as well, further suggests that viral vectors currently in development based upon Herpesviruses, Alphaviruses, Flaviviruses, and Parvoviruses may additionally have unique innate stimulatory properties which could influence vaccine-elicited immune responses (28–33).

Sources and Mechanisms of Differential Innate Immune Stimulation by Adenoviral and Poxviral

Vaccine Vectors

As shown in these studies, viral vectors can differ substantially in their phenotypes of innate immune activation, both within and between viral families. This differential innate immune cytokine and

chemokine elicitation likely occurs through several possible means such as differences in viral structure, cellular tropism, pattern recognition receptor (PRR) access/triggering, and host immune response antagonism. While innate immune stimulation elicited by Ad and Pox vectors described above share some common antiviral characteristics as shown through common induction of IFN- γ and its downstream-signaled chemokines IP-10 and I-TAC, a clear broad bifurcation of responses elicited by Ad and Pox vectors suggests differential mechanisms of innate immune activation between these vector families. This observation is unsurprising given the broad scope of biological differences between Ad and Pox vectors, but it is intriguing that such differences in viral biology would be made dramatically apparent following immunization and stimulation in several systems. While it is possible that non-equivalent innate stimulation elicited by the Ad and Pox regimens on a per-particle basis could play a role in these observed differences, the identities of downstream-signaled chemokines such as IP-10, IL1-RA, MCP-1, ...etc. suggest that the quality and not merely the quantity of innate immune stimulation by Ad and Pox vectors are distinct.

Intriguingly, while innate immune activation elicited by Ad and Pox vectors varied in phenotype relative to the identity and scale of cytokines and chemokines induced, cytokine responses *in vivo* and *in vitro* displayed remarkably similar kinetics for both vector classes (6, 16). For all vectors analyzed, innate immune cytokine responses peaked at approximately 24 hours following vaccination or infection. While it is possible that individual peaks of Ad or Pox vector cytokine levels may occur before or after 24h and not have been observed due to sampling frequency, the general timeframe for cytokine and chemokine responses elicited by these vectors is unlikely to be markedly different than the general 24 h timepoint. This observation suggests that the identity of innate immune cytokines and chemokines elicited following vaccine vector stimulation is influenced largely by the viral vector, while the temporal kinetics of the response are largely determined by the host immune system. Studies which assess the

mechanistic sources of the differences in this early elicitation of cytokines and chemokines are therefore critically important for understanding early adaptive immune response imprinting for future anamnestic recall.

Despite the broad differences in vector biology between Ad and Pox vectors, it is possible to gain insights into the mechanisms by which they elicit innate immune responses through comparison of the sources of differences in innate immune stimulation within each vector family. The vector families utilized in these studies were generated via viral attenuation achieved through several different means (1, 21, 34–37). Ad vectors, as described above, are widely utilized in their replication incompetent form via deletion of Early transcriptional products, in particular the E1A/E1B and E3 proteins. Due to this process, replication-incompetent Ad vector genomic products are largely untranscribed *in vivo*, and do not play a large role in vector biology (38–40). That is to say, Ad vectors likely are largely genetically inert in the process of vaccination. This attenuation essentially creates fully-formed mature Ad particle with all the innate stimulatory PRR's such as dsDNA, but which lack any transcribed products which serve to block host immune induction.

The results shown above provide a model mechanism explaining the differences in innate immune stimulation elicited by Ad vectors, which largely reflect differences in these broad structural properties such as fiber-mediated receptor usage or capsid preference for endosomal escape location. Ad vectors were shown to differ markedly in their innate cytokine and chemokine elicitation profiles, with the CD46-utilizing Ad vectors Ad35 and Ad26 eliciting greater levels of antiviral and proinflammatory cytokines and chemokines than Ad5 (6). This difference largely coincided with primary receptor usage, which is mediated by the Ad fiber protein, as best illustrated by results indicating that switching of viral structural moieties such as fiber or capsid components between Ad5 and Ad35 was able to alter the vectors' resultant innate immune stimulatory phenotypes (41, 42). Importantly, the

pronounced induction of antiviral and proinflammatory cytokines and chemokines by Ad35 and Ad26 was dependent on several PBMC subsets, suggesting that tropism differences mediated by CD46 usage cannot account fully for their observed phenotypes and that a cellular mechanism likely establishes differences in Ad vector innate immune stimulation. Subsequent results showed clear differences in intracellular trafficking patterns between Ad5, Ad35, and Ad26, with Ad35 and Ad26 accumulating to a greater extent in the innate immune sensor-rich late endosome. Although Ad5, Ad35, and Ad26 reached the late endosomal compartment to different degrees, all three were sensitive to a common set of innate immune sensor inhibitors against endosomal acidification, Cathepsin B, and Caspase-1. These differences in intracellular trafficking corresponded to the observed hierarchies of innate immune cytokine and chemokine elicitation observed both in vaccinated rhesus monkeys and humans, as well as in human PBMC (6). These observations suggest a mechanism linking clinically-relevant differences in innate immune cytokine and chemokine elicitation by Ad vectors with vector biological properties influencing the degree to which they access late endosomal innate immune sensors. Furthermore, these results suggest that the means by which Ad vectors can be altered for optimization of their innate stimulatory properties for a given system likely lie in structural alteration of the capsid and fiber proteins, rather than genetic alterations. Additional stimuli may therefore be required to augment Ad vector innate immune stimulation past that observed for the most stimulatory vector. That is to say, that the structural moieties of the CD46-utilizing Ad vectors may represent a “ceiling” of Ad innate stimulatory properties. This point is intriguing and warrants further study through analysis of other Ad vectors for their innate stimulatory properties.

Similar to the replication-incompetent to the Ad vectors, the Pox vectors ALVAC, MVA, and NYVAC are unable to complete their replication cycle in mammalian cells (43). However, these vectors retain the ability to express large portions of their genome during their abortive replication cycles. Due

to many poxviral proteins still being produced during ALVAC, MVA, or NYVAC vaccination or infection, genetic elements in these vectors may contribute to and help shape resultant innate and adaptive immune responses (44)(45)(46). As mentioned above, these vectors possess many intact ORF's encoding decoy cytokine receptors, intracellular signaling inhibitors. Differences in these security protein arsenal functions between Pox vectors, rather than differences in Poxviral structural properties, have been previously shown by several groups to be influential in Poxvirus attenuation and immunogenicity, and likely contribute to the observed differences in innate immune stimulation elicited by ALVAC, MVA, and NYVAC observed in these studies (25–27). This point suggests that the differences in innate stimulation between Pox vectors is due in large part to a genetic component, in contrast to the structural differences being important for Ad vectors. Furthermore, if innate stimulatory properties of Pox vectors are determined by their array of available security proteins, it suggests that the innate stimulatory properties of them are malleable and are able to be further optimized through complementation and/or deletion of security proteins.

Viral Vector Innate Immune Stimulation as Biologic Adjuvants

Adjuvants are substances used to enhance the immune system's response to a desired antigen through stimulation of host immune sensing pathways. However, a challenge to bringing novel adjuvants to use in the clinic is the anticipated cost, regulatory mechanisms, and possible legal liability making their development financially prohibitive (47, 48). As such, a means of adjuvanting immune responses with currently approved biologics would potentially harness the wealth of adjuvanting signaling pathways available while circumventing existing regulatory hurdles. Viral vectors, through stimulation of innate immune danger-sensing pathways are able to serve such an adjuvanting role in vaccines, as has been previously observed in several studies (49, 50). Viral vectors of several different

families and modifications have a long-standing excellent safety profile as shown in both clinical studies as well as ongoing vaccination regimens. Furthermore, biological agents derived from live viruses which inherently possess several PAMP's, viral vectors are inherently immunostimulatory to many PRR's spread throughout the immune system (50–52). The studies presented herein indicate that viral vectors are not only able to serve as basic adjuvants to elicit innate immune stimulation in the proximity of a carried transgene, but may have a greater utility in vaccine-design through their ability to induce several phenotypically distinct types of innate immune activation. They therefore may be able to serve as specific adjuvants to skew adaptive immune responses toward an antigen to a specific set of adaptive immune phenotypes. That is to say, it is possible that viral vectors may serve as a novel class of adjuvants which can be consciously selected to be paired with their cognate antigen to elicit a targeted immune response of a desired phenotype.

As mentioned above, the innate cytokine and chemokine profiles shown in these studies are likely important as they potentially influence/reflect adaptive immune response phenotype formation. Ad5 and Ad5HVR48 vectors have been demonstrated to induce a robust CD8⁺ T-cell response in the peripheral circulation following vaccination as well as efficiently transduce and express large amounts of a desired transgene (1, 8). However, despite the relatively high magnitude of CD8⁺ T-cell responses elicited by Ad5 vectors, these elicited T cells are unable to efficiently control pathogenic HIV/SIV infection, as shown in several studies (3, 53). In particular, the cytotoxic CD8 T cells induced by Ad5 vaccination have been shown to possess a more anergic/exhausted phenotype, suggesting that a robust CD8⁺ T cell response may not necessarily correspond to functional vaccine-elicited immunity against a pathogen (14). In the studies presented herein, Ad5 and Ad5HVR48 were observed to induce relatively little innate immune cytokine and chemokine responses following both vaccination of rhesus monkeys and infection of human PBMC. This lack of innate cytokine elicitation has been independently

reproduced and further shown to allow transgene expression for a greater period relative to Ad35 and Ad28 in a mouse vaccination system (54). Therefore, a possible source of this decreased cellular functionality may be comparatively strong transgene expression and stimulation of T cells without the cognate innate immune activation providing dendritic cell activation and licensing. Particularly, the relatively low levels of antiviral signals via IFN- α 2 and IFN- γ signaling following potent transgene production by an Ad5 vector may serve as a tolerizing, rather than a stimulatory vaccination through lack of sufficient “Signal 3” for efficient memory T cell formation (55). This process would lead to induction of T cell responses which had not received sufficient licensing for cytotoxic potential and therefore become comparatively anergic and exhausted. While perhaps detrimental for vaccination against intracellular pathogens, the tolerizing effect of an Ad5 vaccine however could make it useful for other applications where immune anergy is desired, such as vaccination against allergens or against antigens targeted in autoimmunity.

Similar to Ad5, the CD46-utilizing vectors Ad35 and Ad26 induce a numerically robust CD8⁺ T-cell response following vaccination. However, in contrast to Ad5, these T-cells have a largely more functional phenotype centered on anti-viral immunity (14). Relative to Ad5-induced T-cell responses, they display greater polyfunctionality, expanded anamnestic recall potential, and are more of the effector memory phenotype, corresponding to what is classically thought to represent a predominantly anti-viral adaptive immune response. Importantly, CD8⁺ T cell polyfunctionality and killing potential have also been observed to be important for the control of pathogenic SIV infection, and the increased protection conferred by Ad35 and Ad26 relative to Ad5 in the SIV infection model may reflect their expanded ability to elicit these CD8⁺ T-cell phenotypes (2, 3, 56–58). As shown in these studies Ad35, Ad26, and Ad48 all induce a cytokine milieu of greater anti-viral character relative to Ad5 predominantly centered around IFN- α 2 and IFN- γ and their downstream-signaled cytokines and chemokines. Anti-viral cytokine

signaling, such as through IFN- α has been shown to increase cytotoxic potential by CD8⁺ T cells (59). Therefore, the robust anti-viral character of cytokine and chemokine responses elicited by the CD46-utilizing Ad vectors may play a role in the induction of more functional anti-viral adaptive immune responses by these vectors. Additionally, the role of proinflammatory cytokines in this stimulatory milieu is also possibly important for attracting and activating antigen presenting cells to the site of vaccination and/or draining lymph node, further bolstering the anti-viral stimulatory environment. Importantly, it is unlikely that the anti-viral cytokine and chemokine milieu elicited by Ad35 and Ad26 only influences resultant CD8⁺ T cell phenotypes, and as such its influence on other important cell populations such as T follicular helper cells (T_{FH}), other CD4⁺ T-cell subsets, B cells, and NK cells remains to be determined. The potent anti-viral cytokine and chemokine induction by CD46 Ad's may make them most useful for applications where potent intracellular immunity is required, such as for viral diseases as well as malignancies, and means by which this capacity of the vectors can be augmented warrants further investigation

In contrast to the Ad vectors and as noted above, the poxviral vectors ALVAC, MVA, and NYVAC have been previously shown to induce a balanced CD4⁺/CD8⁺ T cell response following vaccination, rather than a predominantly CD8⁺ response (4, 60). The elicitation of a greater breadth of pro-inflammatory cytokines and chemokines by these vectors *in vivo* may be influential in eliciting this phenotype, however the connections between specific classes of cytokine responses induced by the vectors and the more general immune responses which arise following vaccination with them are less clear than those with Ad vectors. The induction of CD4⁺ helper responses following vaccination is potentially important given the results of the correlations study of the RV144 trial indicating that antibody responses are important in lowering or increasing risk of HIV-1 acquisition (61). The means by which poxviral vectors may stimulate T_H2 immunity or other T_H phenotypes and the possible roles of the

expanded pro-inflammatory cytokine and chemokine repertoire elicited by poxviral vectors *in vivo* may potentially influence this process. Additionally, understanding whether the influence of the observed differences between Poxviral innate cytokine and chemokine responses are made apparent in differential induction of antibody or T-cell phenotypes will be important for future vaccine construction.

Viral vectors represent a potential novel class of biologic adjuvants which may be able to be utilized to skew immune responses towards desired phenotypes. However, the connection between vector-elicited innate immune phenotypes immediately following vaccination and subsequent adaptive immune response formation are complex and currently poorly understood (62). Future experiments focusing on utilizing different vaccine vectors with identical vaccine transgenes and common route of vaccination would allow the exploration and correlation of innate immune cellular and adaptive immune phenotypes to be established. The results presented in these studies are a logical starting point for the exploration of vector influence on vaccine-elicited immune responses. Moreover, these studies suggest that vectors based on viral families other than Ad and Pox may possess distinct innate stimulatory properties. This putative variability in vector family innate immune stimulation, coupled with the observed differences in innate immune stimulation elicited within vector families, could potentially allow both the broad selection and fine tuning of vector innate immune properties for a desired vaccine. Studies *in vivo* utilizing vectors with identical transgenes and comprehensive post-immunization innate and adaptive immune phenotyping would allow such a comprehensive characterization of vaccine vector responses to be obtained, and would be broadly applicable for many applications of viral vector technology.

The results presented herein indicate that viral vectors both within and between viral families can differ profoundly in their innate immune stimulatory properties. Additionally, the mechanisms by which they differ in their innate stimulatory properties can be rooted in different facets of vector

biology such as vector structure or genomic content. These data indicate that viral vectors, rather than being passive shuttles, may play an active role in determining the innate and adaptive immune phenotypes elicited against a target antigen. Moreover, viral vectors can potentially be thought of as a novel class of adjuvanting biologics with a long-standing safety profile which can be modified in a variety of ways to take advantage of the various immune stimulatory phenotypes which have been shown here to exist for Ad and Pox vectors, and which likely exist for other viral vector systems as well. This broad diversity of innate immune stimulatory phenotypes has the potential to allow the adaptation and optimization of viral vectors for a wealth of uses against viral, bacterial, and parasitic diseases as well as applications for targeting of malignancies or induction of allergen tolerance. Further exploration as to the connections between innate stimulatory phenotypes of these vectors and resultant adaptive immune phenotypes will yield exciting insights not only into questions of fundamental immunology, but also into the means why which vaccines elicit immune-mediated protection from disease.

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